A Simple Virus Recovery Assay to detect the Replication-Competent HIV-1 Reservoir in Children

by

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Declaration

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Abstract

Background and Introduction

The early initiation of combined antiretroviral therapy (cART) in children has proved to significantly reduce the morbidity and mortality rate associated with human immunodeficiency virus type 1 (HIV-1) infection. However, even though cART can reduce plasma viral loads to being clinically undetectable, it does not cure HIV-1 infection due to the presence of a latent reservoir where HIV-1 persists as integrated, replication-competent proviruses. For this reason, the search to finding a cure for HIV-1 continues, either by targeting the HIV-1 reservoir directly (eradication approach) or by controlling rebound of viraemia (functional cure approach). To accurately assess the success of such cure strategies, assays to accurately measure the latent reservoir are essential, such as the quantitative viral outgrowth assay (qVOA). In this study, a simple viral outgrowth assay (VOA) was developed that utilises MOLT-4 CCR5+ cells for propagation of HIV-1 as well as a sensitive reverse transcriptase real-time polymerase chain reaction (RT-qPCR) assay for detection of viral outgrowth.

Methods

Two children (patient 337756 and patient 341622) were selected that were part of the post-Children with HIV Early Antiretroviral Therapy randomised trial (CHER) to be assayed for replication-competent virus, with the VOA, from stored peripheral blood mononuclear cells (PBMCs) form as single blood collection, which consisted of stimulation to induce viral release followed by co-culture over 28 days. Both children started therapy at two months of age, were ART suppressed for eight years, although both had a recent episode of viraemia (six months before the time point sampled), and both had total HIV-1 deoxyribonucleic acid (DNA) levels of >50 copies per million cell equivalents at the time point sampled. Patient 337756 was 11 years old and patient 341622 was 10 years old at the time point sampled. A total of 29 million and 21 million PBMCs were assayed for patients 337756 and 341622, respectively. A VOA was implemented and optimised that utilised MOLT-4 CCR5+ cells for HIV-1 expansion and a sensitive, affordable RT-qPCR assay was developed that targets the *integrase* region of *pol* in the HIV-1 genome to detect exponential viral outgrowth. T cells were stimulated by addition of phytohemagglutinin (PHA) and irradiated feeder cells. To

determine the sensitivity of this RT-qPCR assay, it was compared to the traditional, but more expensive p24 antigen enzyme-linked immunosorbent assay (ELISA).

Results

Infectious virus was recovered from two out of three VOA wells in one of the two children (patient 337756). The in-house RT-qPCR assay was able to detect virus on day five for this child while the p24 ELISA only had a positive result on day 14. Interestingly, the second VOA well for the same child had low-level exponential outgrowth with the RT-qPCR assay, but the p24 had no positive signals. This proved that the RT-qPCR assay was more sensitive in detecting viral outgrowth compared to the p24 ELISA. The third VOA well for this patient had weak ribonucleic acid (RNA) signal on day 14 as well as DNA signal on day seven. The child who had no infectious virus recovery from stored PBMCs (patient 341622) also had a single instance of weak RNA signal on day 14 in one of the two VOA wells assayed. The single instances of RNA release might have been non-infectious, inducible virus that got released after stimulation. HIV-1 DNA detected in the supernatant were cell derived and may therefore indicate apoptosis or lysis of infected cells.

Conclusion

The VOA implemented in this project proved to be more practicable by using MOLT-4 CCR5+ cells compared to the gold standard qVOA that makes use of cluster of Differentiation 8 (CD8+)-depleted lymphoblasts from different donors. The RT-qPCR assay also proved to be more sensitive than the p24 ELISA. Although, by streamlining and optimising this assay even more, it could be more valuable for future studies in evaluating HIV-1 eradication strategies. Nevertheless, this VOA could be a valuable tool in studying the success of latency reversing agents (LRAs) in children on suppressive cART.

Opsomming

Agtergrond en Inleiding

Die vroeë inisiasie van gekombineerde antiretrovirale terapie (ART) in kinders het met betrekking tot menslike immuniteitsgebreksvirus tipe 1 (MIV-1) infeksie, bewys om die morbiditeit en sterftesyfer aansienlik te verminder. Alhoewel ART plasma virale ladings kan verlaag tot klinies onaantoonbaar, is dit nie instaat om MIV-1 te genees nie as gevolg van die teenwoordigheid van ‘n latente reservoir waar MIV-1 voortbestaan as geïntegreerde, replikasiebevoegde provirusse. Daarom gaan die soektog vir ‘n geneesmiddel vir MIV-1 voort, óf deur die MIV-1 reservoir direk te teiken (uitroeiingsbenadering), óf deur die herstel van viremie te beheer (funksionele genesingsbenadering). Om die sukses van sulke genesingstrategieë akkuraat te beoordeel is dit noodsaaklik om die latente reservoir akkuraat te meet met behulp van toetse soos die kwantitatiewe virale uitgroei toets (kVUT). In dié studie was ‘n eenvoudige virale uitgroei toets (VUT) ontwikkel wat MOLT-4 CCR5+ selle gebruik het vir die replisering van MIV-1, asook ‘n sensitiewe tru-transkriptase intydse kwantitatiewe polimerase kettingreaksie (TT-kPKR) toets om virale uitgroei te meet.

Metodes

Twee kinders (pasiënt 337756 en pasiënt 341622) is gekies wat deel was van die post-kinders met MIV Vroeë antiretrovirale terapie (CHER) gerandomiseerde studie. ‘n 28-dag VUT is gedoen om te toets vir replikasie-bevoegde virus in die kinders, van gestoorde mononukleêre selle van perifere bloed (MSPB) wat gestimuleer is om virus vry te stel gevolg deur mede-kultuur. Albei kinders was twee maande oud toe ART geïnisieer is, hulle was albei agt jaar lank virologies onderdruk met behulp van ART, alhoewel albei het n onlangse episode van viremie gehad (ses maande voor die monster wat getoets was), en albei het totale MIV-1 deoksiribonukleïensuur (DNS) vlakke van >50 eksemplare per miljoen selekwivalente op die gegewe tydstip gehad. Pasiënt 337756 was 11 jaar oud en pasiënt 341622 was 10 jaar oud op die gegewe tydstip. Altesaam 29 miljoen en 21 miljoen MSPB is onderskeidelik vir pasiënte 337756 en 341622 getoets. ‘n VUT was geïmplementeer en geoptimiseer wat MOLT-4 CCR5+ selle gebruik het vir MIV-1 uitbreiding, en ‘n sensitiewe bekostigbare TT-kPKR toets was ontwikkel wat die integrasegeen van pol in die MIV-1 genoom teiken om eksponensiële virale uitgroei op te spoor. T-selle was gestimuleer deur

fitohemagglutinien (PHA) en bestraalde voerselle. Om die sensitiwiteit van hierdie TT-kPKR toets te bepaal was dit vergelyk met die tradisionele, maar duurder p24 antigeen ensiemgekoppelde immunosorbent toets (ELISA).

Resultate

Infektiewe virus is in twee van drie VUT putte in een van die twee kinders ontdek (pasiënt 337756). Die binnenshuis TT-kPKR toets kon virus opspoor op dag vyf vir hierdie kind terwyl die p24 ELISA eers op dag 14 ‘n positiewe resultaat gehad het. Dit was interessant dat die tweede VUT put vir hierdie kind lae vlakke eksponensiële groei gehad het met die TT-kPKR toets, maar daar was geen positiewe p24 resultate nie. Dit het bewys dat die TT-kPKR toets meer sensitief was om virale uitgroei op te spoor as die p24 ELISA. Die derde VUT put vir hierdie pasiënt het ‘n swak ribonukleïensuur (RNS) sein op dag 14 gehad, sowel as DNS sein op dag sewe. Die kind wat geen infektiewe virus lading gehad het nie van gestoorde MSPB (pasiënt 341622), het op dag 14 ook ‘n swak RNS sein in een van die twee VUT putte gehad. Die enkele gevalle van RNS vrystelling was moontlik nie-aansteeklike, induseerbare virus wat na stimulasie vrygestel is. MIV-1 DNS wat in die supernatant opgespoor was, was heel moontlik van selle en kan dus dui op apoptose of lise van geïnfekteerde selle.

Gevolgtrekking

Die VUT wat in hierdie projek geïmplementeer is, blyk om meer prakties te wees deur die gebruik van MOLT-4 CCR5+ selle in vergelyking met die standaard kVUT wat gebruik maak van ‘n Groep Uitgedifferensieerde Limfoblaste van Differensiasie 8 (CD8+) van verskillende skenkers. Die TT-kPKR is ook bewys om meer sensitief te wees as die p24 ELISA. Alhoewel, deur die toets selfs verder te verbeter en te optimiseer kan dit waardevol wees vir toekomstige studies waarin MIV uitwissing-strategieë geëvalueer word. Nietemin, hierdie VUT kan ‘n waardevolle middel wees om die sukses van latensie omkeermiddels (LRAs) in kinders wat op ART is te bestudeer.

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Research Outputs

Below is a list of research outputs based on work done before, but only presented during the time this master's project was completed.

National Conference attendance:

Poster Presentation:

Van Zyl, C., Veldsman, K.A., Van Zyl, G.U. 2020. **A Sensitive Assay to detect all HIV-1 DNA in Infected Cells.** *Virology Africa 2020 Conference*, Cape Town, SA

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List of Abbreviations

3TC	Lamivudine
A	Adenine
ABC	Abacavir
AIDS	Acquired immunodeficiency syndrome
APD	Average Pairwise Distance
ART	Antiretroviral therapy
ARVs	Antiretroviral drugs
ATRA	*All-trans* retinoic acid
AZT	Zidovudine
BB515	Brilliant™ Blue 515
Bp	Base pairs
CAD-SGS	Cell associated DNA single genome sequencing assay
cART	Combination antiretroviral therapy
CCR5+	C-C chemokine receptor type 5
CD	Cluster of Differentiation
cDNA	Complimentary DNA
CHER	Children with HIV Early Antiretroviral Therapy randomised trial
CPE	Cytopathic effect
CRFs	Circulating recombinant forms
Ct	Cycle threshold
CTLs	Cytotoxic T-lymphocytes
CXCR4+	C-X-C chemokine receptor type 4
DDI	Didanosine
ddPCR	Droplet digital polymerase chain reaction
ddT	Dideoxy Thymine
DEPC	Diethyl pyrocarbonate
DEVO	Digital ELISA Viral Outgrowth
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
DRC	Democratic Republic of the Congo
DTT	Dithiothreitol

E. *coli*	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EFV	Efavirenz
ELISA	Enzyme-linked immunosorbent assay
Env	Envelope
FBS	Foetal Bovine Serum
FDA	Food and Drug Administration
GI	Gamma-irradiated
Gp	Glycoprotein
HAART	Highly active antiretroviral therapy
HCl	Hydrogen chloride
HDACi	Histone deacetylate inhibitors
HI	Heat-inactivated
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HMTi	Histone methyltransferases inhibitors
HPPMCS	HIV Paediatric Prognostic Markers Collaborative Study
HREC	Health Research Ethics Committee
HRP	Horseradish peroxidase
HS	High Sensitivity
iCAD	Integrase cell associated total HIV-1 DNA quantitative assay
ID	Identity
IL	Interleukin
INDELS	Small nucleotide insertions or deletions
IPDA	Intact proviral DNA assay
IPTG	Isopropyl-beta-D-thiogalactopyranoside
ISTIs	Integrase strand transfer inhibitors
IUPM	Infectious units per million cells
kb	Kilobases
KCl	Potassium chloride
LB	Luria Broth
LPV/r	Lopinavir-ritonavir
LRAs	Latency reversing agents
LTR	Long terminal repeat
M	Major

MCS	Multiple cloning site
MFI	Median fluorescent intensity
mRNA	Messenger RNA
ms	Multi-spliced
MS-VOA	Multiple stimulation viral outgrowth assay
MTCT	Mother-to-child transmission
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
N	Nonmajor and nonoutlier
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Hydrogen
NCBI	National Center for Biotechnology Information
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIAID	National Institute of Allergy and Infectious Diseases
NIH	National Institutes of Health
NNRTIs	Non-nucleoside reverse transcriptase inhibitors
NRT	No-reverse transcriptase
NRTIs	Nucleoside-analog reverse transcriptase inhibitors
NSP	National Strategic Plan
NTC	No template control
NVP	Nevirapine
O	Outlier
OD	Optical density
OPD	Ortho-phenylenediamine-HCl
P	“Pending the identification of further human cases”
PBMCs	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PE	Phycoerythrin
PHA	Phytohemagglutinin
PIC	Pre-integration complex
PIs	Protease inhibitors
PMA	Phorbol 12-myristate 13-acetate
PMT	Photomultiplier tubes
qPCR	Real-time quantitative polymerase chain reaction
qVOA	Quantitative viral outgrowth assay
RCF	Relative centrifugal force

RFU	Relative fluorescence units
RNA	Ribonucleic acid
rpm	Rotations per minute
RPMI	Roswell Park Memorial Institute
RT	Reverse transcriptase
RT-qPCR	Reverse transcriptase real-time polymerase chain reaction
SCA	Single-copy assay
Simoa	Single molecule array, ultra-sensitive p24 assay
siRNAs	Small interfering RNAs
SOC	Super Optimal broth with Catabolite repression
STCM	Super T cell medium
TC	Tissue culture
TCGF	T cell growth factor
TDR	5 mM Tris-HCl, 100 mM DTT, 10 000 units/mL recombinant RNasin ribonuclease inhibitor
TGS	Transcriptional gene silencing
TILDA	Tat/rev Induced Limiting Dilution Assay
TLR	Toll like receptors
UNAIDS	Joint United Nations Programme on HIV/AIDS
URFs	Unique recombinant forms
us	Unspliced
UV	Ultraviolet
V	Visit
VOA	Viral Outgrowth Assay
WCBS	Western Cape Blood Service
X-Gal	5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside
ZFN	Zinc-finger nucleases

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Chapter 1

Literature Review

1.1 Introduction

In the year 2019, 38 million people across the world were living with human immunodeficiency virus (HIV). Of these 38 million people, 1.7 million people became infected with the virus in 2019, only 25.4 million people were accessing antiretroviral therapy (ART) and 690 000 people died from acquired immunodeficiency syndrome (AIDS) related illnesses. Of these 38 million people, 1.8 million were children of between the ages zero to 14 years (Joint United Nations Programme on HIV/AIDS (UNAIDS), 2020). The Joint United Nations Programme on HIV/AIDS (UNAIDS) have set a target to help end the AIDS epidemic by 2030, the 90-90-90 target. This target aimed to achieve the following by the year 2020: 90% of all people living with HIV would know their status, 90% of all people with diagnosed HIV infection would receive sustained ART and 90% of all people receiving ART would have viral suppression. Although many countries are making progress in achieving this target, there are still a lot of people living with HIV without knowing they are infected and a lot of people without access to ART (UNAIDS, 2018). Combination antiretroviral therapy (cART) is able to reduce HIV viral loads to being clinically undetectable (Perelson et al., 1997), but cART does not eliminate dormant replication-competent HIV that persists in resting Cluster of Differentiation 4 (CD4+) T cells, known as the reservoir (Finzi et al., 1997). Therefore, the reservoir is the main barrier in achieving a cure for HIV-infection and assays to examine this reservoir is of vital importance in the cure field. This research project aimed to improve on an existing assay to estimate the size of the latent HIV-1 reservoir in children living in South Africa.

1.2 HIV-1 in South Africa

South Africa has the biggest HIV epidemic in the world, with 7.7 million people living with HIV in the year 2018. There was a 20.4% prevalence of HIV infection in adults of between the ages 15-49 years, there were 240 000 new infections and 71 000 AIDS-related deaths.

In 2018, 260 000 children (zero to 14 years old) were living with HIV in South Africa. Of the total number of adults and children living with HIV, 71% of adults and 47% of children were on ART (Avert, 2018). Children and adolescents (zero to 17 years old) are also affected by HIV through the loss of parents or guardians and family members. This makes them more vulnerable to HIV because of economic and social insecurities. South Africa's National Strategic Plan (NSP) 2017-2022 aims to renew the focus on children by putting emphasis on eliminating new infections and building resilience in families (South African National AIDS Council, 2017).

Human immunodeficiency virus can be divided into two types: HIV-1 and HIV-2. Within HIV-1, there are four groups: major (M), outlier (O), nonmajor and nonoutlier (N), and "pending the identification of further human cases" (P). Within HIV-2, there are groups A to H. The most prevalent HIV-1-infection worldwide is caused by group M. Group M is further divided into 10 subtypes (A to D, F to H and J to L) and five sub-subtypes (A1 to A3, F1, and F2) (Hemelaar, 2013). Subtype L was recently discovered in the Democratic Republic of the Congo (DRC) (Yamaguchi et al., 2020). Within South Africa, HIV-1 subtype C remains the dominant subtype (Hemelaar et al., 2011) (Avert, 2019) (Matume et al., 2020).

A genetic sequence that carries regions from two distinct parental strains are known as recombinant forms (Song et al., 2018). There are two types of recombinant forms: circulating recombinant forms (CRFs), which is when recombinant HIV-1 genomes have infected three or more epidemiologically unrelated individuals and unique recombinant forms (URFs), which is a recombinant form that has no evidence of ongoing transmission (Robertson, 2000). A total of 102 distinct CRFs have been described thus far (LOS ALAMOS, 2020).

1.3 Statistics and Characteristics of Paediatric HIV-1 Infection

Most paediatric HIV-1 infections occur due to mother-to-child transmission (MTCT). In the year 2018, 88% of pregnant women were tested for HIV and 87% of women living with HIV were receiving antiretroviral drugs (ARVs) to prevent MTCT. The number of children born to HIV positive mothers declined from 28 000 in 2010 (UNAIDS, 2014) to 14 000 in 2018, however, this was still high and efforts to reduce this even more needs to be examined ("AIDSinfo | UNAIDS", 2020).

In the absence of treatment, progression to AIDS or death usually happens at a median time of 10 years after infection in adults, while 20% to 30% of infants die within the first year of life, even in the case of having a high percentage of CD4+ T cells (Tobin & Aldrovandi, 2013). It has been shown by the HIV Paediatric Prognostic Markers Collaborative Study (HPPMCS) (a meta-analysis of data from untreated HIV-infected children in the United States and Europe) that the risk of death increased by a factor of six for a one year old child that has high CD4+ counts, compared to a five year old child with high CD4+ counts (Dunn et al., 2003).

The kinetics of HIV-1 ribonucleic acid (RNA) plasma viral load also differs remarkably between adults and infants. In adults, the initial viral load spike is rapid, followed by a 100 to 1000-fold decrease in HIV-1 viral copies where a relative stable ‘set point’ is reached within weeks following infection. The viral load in infants increases 10-fold within the first few months of infection to much higher levels to those in adults and it declines slowly and does not reach a ‘set point’ until five years of age (Mcintosh et al., 1996) (Tobin & Aldrovandi, 2013).

The reason for these differences in disease progression and in the plasma viral load kinetics between adults and infants is due to the differences in the immune systems. Infants’ immune systems are still developing which includes the fast expansion of CD4+ T cells, which in turn leads to a high concentration of these cells. Another factor that could contribute to the high level of viraemia seen in young, untreated children are the shared human leukocyte antigen (HLA) alleles between mother and child which preselects the virus for fitness in the infant (Pillay et al., 2005) (Thobakgale et al., 2009).

1.4 HIV-1 Persistence

1.4.1 The HIV-1 lifecycle and the role of antiretroviral therapy

The main targets of the immune system for infection with HIV-1 are: CD4+ T cells (also known as helper T cells), monocytes, macrophages, and dendritic cells (Cunningham et al., 2010). Although, the main cell type known to be infected is the CD4+ T helper cells due to the high binding affinity of the HIV-1 viral envelope (Env)-glycoprotein to the CD4+ receptor on the CD4+ T cell’s membrane (Berger et al., 1999).

An overview of the HIV-1 life cycle can be seen in Figure 1.1. The first stage in the HIV-1 lifecycle involves the binding of the virus's Env glycoprotein (gp) 120 to the CD4+ receptor and the C-C chemokine receptor type 5 (CCR5+) or C-X-C chemokine receptor type 4 (CXCR4+) co-receptors of the target cell. The virus will then fuse with the host cell membrane. This fusion of the virus to the host cell membrane is mediated by HIV-1 Env gp41. Initially it was thought that once HIV-1 fused with the host cell membrane, uncoating followed which then releases the RNA and proteins into the host cell's cytoplasm. However, research has been done in trying to understand the timing, localisation and mechanism of uncoating as it is poorly understood. Recent work has found that there is initial partial uncoating of the HIV-1 capsid shell in the host cell's cytoplasm, with full uncoating occurring at the nuclear pore after completing reverse transcription (Ambrose & Aiken, 2014) (Cosnefroy et al., 2016). Reverse transcription converts the HIV-1 RNA into deoxyribonucleic acid (DNA), which is known as the pre-integration complex (PIC). The PIC is carried into the nuclease of the host cell where it is integrated into the host's DNA through the enzyme integrase. The virus now exists as a provirus. Cellular components from the host cell are used to transcribe the integrated provirus into more than 40 different viral messenger RNA (mRNA) transcripts that are derived from alternative splicing of the primary RNA transcript. At first, multi-spliced (ms)-RNA transcripts are produced, which encodes for the HIV-1 regulatory proteins Tat, Rev and Nef. Then a shift happens toward the production of unspliced (us) and single-spliced transcripts. Nuclear export of us-genomic RNA is facilitated by the HIV-1 Rev protein (Suhasini & Reddy, 2009). The us-RNA is translated into the structural precursor polyproteins for the *gag* and *pol* gene products, and it is incorporated into virions as genomic RNA. The different single-spliced RNAs are translated into the envelope proteins and other accessory proteins, such as Vif, Vpu and Vpr, which will assemble into budding immature virions (Massanella & Richman, 2016). The new immature virions bud off and are released, with each virion packaging two RNA strands. The virions will then mature through cleaving of the structural polyprotein with the protease enzyme to form mature Gag proteins which will result in the production of new infectious virions (Barre-Sinoussi et al., 2013) (Herrera-Carrillo & Berkhout, 2015).

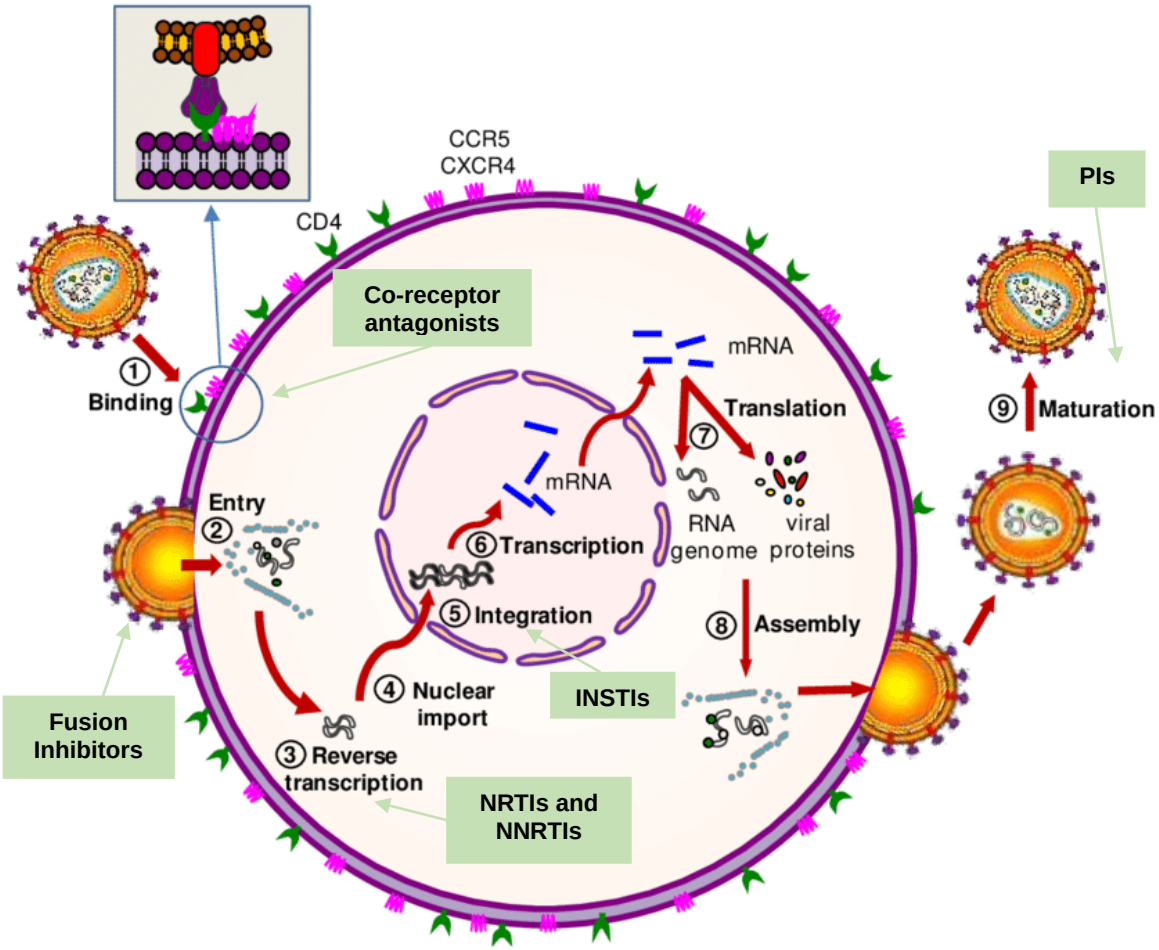

Figure 1.1: The HIV-1 life cycle and the different steps targeted by ARVs

The individual steps of the HIV-1 life cycle are indicated by numbers (number one to nine). Ambrose and Aiken (2014) as well as Cosnefroy et al. (2016) have shown that there is initial partial uncoating of the HIV-1 capsid shell in the host cell's cytoplasm (from step two to three), with full uncoating occurring at the nuclear pore after completing reverse transcription (step four). The steps of the life cycle targeted by the antiretroviral drugs (ARVs) can be seen in green (NRTIs: nucleoside-analog reverse transcriptase inhibitors. NNRTIs: non-nucleoside reverse transcriptase inhibitors. INSTIs: integrase strand transfer inhibitors. PIs: protease inhibitors). Picture sourced from Herrera-Carrillo and Berkhout (2015) Small changes were made to the picture.

Infected CD4+ T cells usually die through one of the following mechanisms: pyroptosis of infected cells (Doitsh et al., 2014), apoptosis of uninfected cells that are in close contact to the infected cells (Garg et al., 2012), or killing of the infected CD4+ T cells by CD8+ cytotoxic T lymphocytes (CTLs) and natural killer cells that recognise the infected cells. This causes CD4+ T cell levels to drop to a below the normal level, causing the body to become more susceptible to opportunistic infections which will eventually lead to AIDS, but this is where ARVs come in.

There are six distinct classes of Food and Drug Administration (FDA) approved drugs available that target different steps in the HIV-1 life cycle to inhibit replication: NRTIs, NNRTIs, INSTIs, PIs, fusion inhibitors and coreceptor antagonists (Coffey & Volberding, 2012) (Arts & Hazuda, 2012). The steps that all these drugs target can be seen in green in Figure 1.1.

By administering cART, which consists of different classes of the drugs (usually three ARVs against a minimum of two distinct molecular targets), viral replication is suppressed and the plasma HIV-1 viral loads are reduced to below the limit of detection of commercially available assays. This helps preserve the immune system which will lead to a better quality of life and a longer life expectancy (Autran et al., 1997) (Lederman et al., 1998).

1.4.2 Establishment of latency in HIV-1 infection

Even though cART is successful in blocking certain steps in the HIV-1 life cycle, it is not able to cure HIV-1 because of the presence of a latent viral reservoir in resting memory CD4+ T cells (Finzi et al., 1997). Siliciano et al. (2003) have found that this latent reservoir has an extremely long half-life of 44 months which results in lifelong ART being necessary. The latent reservoir is established when HIV-1 infects fully or partially activated CD4+ T cells that return to a resting memory state as part of the normal physiological process after encountering cognate antigens (Siliciano & Greene, 2011). Therefore, latent integrated proviruses are found mostly in different subsets of resting memory CD4+ T cells, and at the lowest concentration in naïve CD4+ T cells (Chun et al., 1997) (Hiener et al., 2017). Macrophages show resistance to viral cytopathic effect (CPE) of HIV-1, and therefore also support HIV-1 persistence in certain anatomical sanctuaries of the human body (Kim & Siliciano, 2016). Latent infected cells are not targeted by the immune system or ART, because the resting memory CD4+ T cells do not express the proteins encoded by the viral genes and HIV-1 persists as an integrated provirus that is not actively transcribed (Ruelas & Greene, 2013). There have been studies that showed that homeostatic and antigen-induced proliferation of latently infected cells (Kim & Siliciano, 2016) as well as ongoing replication in sanctuaries (like the brain, genital tract, gut mucosa and lymph nodes) where drug levels are suboptimal together with increased immune activation and inflammation, supports HIV-1 persistence during cART (Fletcher et al., 2014) (Massanella et al., 2016) (Huang et al., 2016). Proviruses in latently infected CD4+ T cells can either be

induced to release replication-competent virus after one round of T cell stimulation, which is referred to as induced proviruses or if they do not release replication-competent viruses they are referred to as non-induced proviruses. Ho et al. (2013) have found that most non-induced proviruses are defective and contain large internal deletions, inactivating G-to-A nucleotide substitutions introduced by APOBEC3G, packaging signal deletions, missense mutations and small nucleotide insertions or deletions (INDELS). These defective viruses will not produce infectious viruses, however a small percentage of non-induced proviruses contain intact genomes and therefore contribute to the latent reservoir. The HIV-1 replication-competent reservoir can become activated when ART is interrupted or when stimulated by an antigen (Siliciano & Greene, 2011) which will result in a rapid viral rebound (Chun et al., 1999). The introduction of early cART can limit the size of the latent reservoir, since the latent reservoir is established very early after infection (Melkova et al., 2016), but not eradicate it. The latent reservoir is therefore seen as a major barrier to eradicating HIV-1.

1.4.3 Persistence markers and assays used to study HIV-1 persistence

The latent reservoir persists under suppressive cART, and while this reservoir is undetectable using commercially available assays that measure HIV-1 RNA in plasma (HIV-1 viral load), HIV-1 persistence can still be determined by: measuring markers of immune hyperactivation that persists, and measuring cell-associated HIV-1 RNA in peripheral blood and tissues like the lymph nodes, tonsils, gut and testes, and activating the latent cells with latency reversing agents (LRAs) in order to release replication-competent HIV-1 (Melkova et al., 2016).

The quantitative viral outgrowth assay

The gold standard for measuring the true latent reservoir is the quantitative viral outgrowth assay (qVOA), as this assay measures replication-competent virus. The assay starts by purifying resting CD4+ T cells (CD69+, CD25+ and HLA-DR+CD4+ T cells are excluded) (Massanella & Richman, 2016) from patient peripheral blood mononuclear cells (PBMCs) infected with HIV-1 that is on suppressive ART. The resting CD4+ T cells are plated in fivefold serial dilutions (the cell input usually ranges from 1 x 10^6 to 320 cells/well) followed by the addition of phytohemagglutinin (PHA) (which is a lectin) along with irradiated allogeneic PBMCs to induce global T cell activation. Since the PBMCs contain macrophages and dendritic cells which act as antigen-presenting cells and enhances the PHA-driven

activation of the T lymphocytes, successful activation is ensured (Siliciano & Siliciano, 2005). To expand the virus released from the patient cells to reach detectable levels, either CD8+ depleted donor lymphoblasts from HIV-1 negative donors can be used or the process can be simplified by using a continuously proliferating MOLT-4 CCR5+ cell line. The patient CD4+ T cells are then co-cultured with the CD8+ depleted donor lymphoblasts or MOLT-4 CCR5+ cells for two to three weeks. Culture supernatants are analysed for free virus by using an enzyme-linked immunosorbent assay (ELISA) for HIV-1 p24 antigen or using a highly sensitive reverse transcriptase real-time polymerase chain reaction (RT-qPCR) assay that will measure the HIV-1 RNA in the supernatant as early as seven days after activation (Laird et al., 2013).

The qVOA has the following disadvantages, it requires large blood volumes of the HIV-1-infected patients' to isolate sufficient resting purified CD4+ T cells and to be able to plate them in serial dilutions. The qVOA is also very material-, labour- and cost-intensive. It also requires culturing time of two to three weeks (for media changes and maintenance of the cells in culture) (Siliciano & Siliciano, 2005). The assay also underestimates the size of the latent reservoir, as only one round of stimulation is performed, and it has been shown that stimulation of the isolated cells more than once could lead to more virus being recovered. The actual size of the latent reservoir might be 60 times higher than what is observed in the qVOA, this complicates the effort to cure HIV-1 using latency reversal (Bruner et al., 2015). Different laboratories also employ different methods to quantify replication-competent virus with the qVOA, such as using different cell subsets from HIV-1-infected patients (resting versus total CD4+ T cells), using different stimulation methods and using different donors for expansion of HIV-1 which causes great variability between assays. This can be improved by using MOLT-4 CCR5+ cells (Massanella & Richman, 2016).

Furthermore, when measuring the latent reservoir, it is important to only detect stably integrated proviruses and not extrachromosomal HIV-1 DNA forms. These extrachromosomal HIV-1 DNA forms are unstable and replication-defective. Stevenson et al. (1990) have shown that resting CD4+ T cells from viraemic patients contained mostly HIV-1 DNA that were linear and unintegrated which represents the end product of reverse transcription before integration. When performing the qVOA on viraemic patients, the unintegrated viral DNA is integrated and transcribed which leads to infectious virus being

released after stimulation. There have been studies that suggest that this linear, unintegrated form of the viral genome is labile in the absence of integration (Zack et al., 1990) (Pierson et al., 2002). For this reason, cells that contain unintegrated HIV-1 DNA should not be considered part of the latent reservoir. Blankson et al. (2000) demonstrated that labile, unintegrated HIV-1 DNA in recently infected cells decay, and after six months the amount of cells detected with the viral outgrowth assay (VOA) drops to a steady plateau after ART has been initiated. Therefore, it is important to note that because of the reasons just mentioned, the qVOA should not be performed on viraemic or untreated patients or on patients who have been on therapy for less than six months when wanting to determine the size of the latent reservoir. Despite all these disadvantages, the qVOA has the advantage of not detecting defective proviruses, as it only measures replication-competent proviruses in patients undergoing suppressive cART (Ho et al., 2013).

The Tat/rev Induced Limiting Dilution Assay

An assay was developed by Procopio et al. (2015) that detects *tat*/*rev* ms-RNA in total CD4+ T cells that have been maximally activated to measure the frequency of cells that harbour inducible virus known as the Tat/rev Induced Limiting Dilution Assay (TILDA). The assay works by stimulating the CD4+ T cells from ART suppressed patients followed by distributing the cells (in a limiting dilution format) in 24 replicate wells in a 96-well plate. Nested polymerase chain reaction (PCR) is directly performed without having to extract the RNA to amplify the *tat*/*rev* transcripts. Maximum likelihood methods are used to determine the frequency of cells producing ms-RNA. Some advantages of the TILDA include that it does not rely on the amplification of viral replication, it does not require extraction of RNA, it requires small blood volumes (10 mL), and the TILDA is well suited for longitudinal clinical studies in that the 96-well plates that contain the serially diluted cells can be stored at -80°C for future quantification. The production of *tat*/*rev* ms-RNA, does not indicate replication competence but just transcriptional activation of at least partially intact proviruses. Therefore, the size of the true latent reservoir may be overestimated with the TILDA, although less than compared to real-time polymerase chain reaction (qPCR) assays. Dhummakupt et al. (2020) showed that there are differences in the inducibility of the latent HIV-1 reservoir in perinatal and adult infections using TILDA. They saw that a single round (12 hours) of CD4+ T cell stimulation with Phorbol 12-myristate 13-acetate (PMA)/ionomycin maximally activated T cells and led to proviral expression with ms-HIV-1 RNA production in

adults. The latent reservoir was slower to reactivate and of lower magnitude in children using these same conditions. Using an enhanced TILDA which added PHA and lengthening the time to 18 hours increased proviral expression in the children, but not in adults. These findings supported the theory that there are differences in kinetics of baseline immune activation and latency reversal in perinatal compared to adult infections.

Real-time PCR for HIV-1 DNA

There are also PCR-based assays which are commonly used to measure persistent HIV-1. General advantages of PCR-based assays over the qVOA are that these methods provide a faster and easier way to study the latent reservoir and the PCR-based assays can be applied to various cell types. The most common PCR assays to measure the latent reservoir is a qPCR that targets cell-associated HIV-1 DNA in infected cells (Cillo et al., 2013) (Rouzioux et al., 2014). These methods involve extraction of DNA from the desired cell populations that have been isolated from peripheral blood. These assays use primers located in conserved regions of the HIV-1 genome and qPCR is performed on the extracted DNA. A standard curve is constructed with a known copy number of proviral DNA (from a plasmid standard containing an infectious clone of HIV-1) so that the number of infected cells can be calculated. To obtain an estimate of the total number of cells in the sample, a cellular gene that is present in two copies per diploid genome is quantified by qPCR. The total number of cells present in the sample, together with the proviral DNA copy number, gives an estimate of the frequency of cells harbouring HIV-1 DNA (Bruner et al., 2015). These assays tend to overestimate the size of the latent reservoir, as most of the proviruses have mutations which renders them defective (Ho et al., 2013).

Droplet digital PCR for HIV-1 DNA

The droplet digital PCR (ddPCR) technique is another PCR method that measures HIV-1 DNA in CD4+ T cells (Strain et al., 2013) as well as in PBMCs (Eriksson et al., 2013). In comparison with qPCR that measures relative quantification of HIV-1 DNA derived from a standard curve, this method allows for absolute quantification of HIV-1 DNA.

Alu-PCR for integrated HIV-1 DNA

To be able to distinguish between linear, unintegrated HIV-1 DNA and integrated HIV-1 DNA, Alu-PCR can be used on both CD4+ T cells and PBMCs (Brady et al., 2013). This method is able to amplify integrated HIV-1 DNA by using a primer that targets the Alu elements that are present in high copy numbers in the human genome and another primer that targets the *gag* gene in the HIV-1 genome. This is followed by performing nested qPCR with a second set of primers that targets the long terminal repeat (LTR) region in the HIV-1 genome. For this assay it is important to include controls in which the Alu primer is excluded to prove that the observed signal comes from integrated HIV-1 DNA. To justify differential amplification of proviruses integrated at different distances from an Alu element, a standard curve that contains a mixture of DNA from cells with different HIV-1 integration sites is used. In the case of proviruses being too far from an Alu sequence to be detected, a correction factor is used. Eriksson et al. (2013) found that there was a strong correlation when comparing Alu-PCR with ddPCR for HIV-1 DNA in patients that are undergoing suppressive ART. This indicated that the HIV-1 DNA was integrated for patients undergoing suppressive ART.

Intact proviral DNA assay

Bruner et al. (2019) recently described a quantitative PCR assay to quantify likely intact and defective proviruses. This assay is referred to as the intact proviral DNA assay (IPDA) and as it is more affordable and scalable compared to the qVOA, it could be used to monitor the impact of curative interventions on intact proviruses.

Single-copy assay

Even when patients are on suppressive cART, it is still possible for residual viraemia to persist in low levels in the plasma (Sahu et al., 2010). A highly, sensitive single-copy assay (SCA) has been developed by Palmer et al. (2003). The SCA is a RT-qPCR that measures viral release from CD4+ T cells or from other stable reservoirs by using primers that are located in a conserved region of gag in the HIV-1 genome and it can detect HIV-1 RNA down to 1 copy/mL of plasma (Palmer et al., 2003). However, recently it has been shown that residual viraemia does not necessarily imply replication-competence, as clones with intact or partially intact proviruses could release virus. Some of these released viruses are

defective (Imamichi et al., 2020). Therefore, the SCA might not be the best assay to measure ongoing replication in patients on suppressive cART. Table 1.1 shows a summary of the assays available to measure the latent reservoir.

Table 1.1: Assays for measuring the HIV-1 latent reservoir

Assay name	Description	Strengths	Limitations
Qvoa	Replication-competent virus induced after one round of T cell activation.	Only quantifies replication-competent proviruses.	Expensive. Requires large blood volumes (120-180 mL). Time consuming. Does not detect all proviruses that pose a barrier to cure. Great variability due to donor variability (improved by using a cell line such as MOLT-4 CCR5+).
TILDA	Tat/rev ms-RNA in total CD4+ T cells.	Less time consuming (no extended cell culture time needed). Small blood volumes (10 mL). Well suited for longitudinal studies.	Overestimates the size of the latent reservoir as defective viruses are detected.
qPCR for HIV-1 DNA	Total proviral DNA.	Easy and quick.	
ddPCR for HIV-1 DNA	Total proviral DNA.	Provides absolute quantification rather than relative.	
Alu-PCR for integrated HIV-1 DNA	Integrated proviral DNA.	Able to measure integrated proviruses.	
SCA	Residual viraemia.	Useful to measure ongoing virus production.	
IPDA	Likely intact and defective proviruses.	Able to distinguish between likely intact and defective proviruses.	Not all defects are detected-only approximation of intact reservoir.

1.5 The Impact of Treating Children Early

Initiating cART early (within the first few months of life) has shown to control HIV-1 replication efficiently, preserve the immune function (Luzuriaga et al., 1997) (Luzuriaga et al., 2000) and therefore, to noticeably reduce HIV-1 related mortality (Violari et al., 2008). For these reasons, it is recommended globally to diagnose and start therapy as soon as possible (WHO, 2018).

A study done by Luzuriaga et al. (2004) showed that infants who started ART before three months of age were able to suppress HIV-1 better. Evolution within the *gag* and *nef* genes of HIV-1 have been reported within the first three months of life in untreated infants (Sanchez-Merino et al., 2005). Persaud et al. (2007) have shown that early effective highly active antiretroviral therapy (HAART) is associated with long-term absence of detectable adaptive immune responses to HIV-1 (HIV-1 specific humoral and cellular immune responses), long-term preservation of limited HIV-1 evolution and long-term control of virus replication in children. However, even with successful early HAART, replication-competent virus from resting CD4+ T cells persist in most children. They were able to recover replication-competent virus from 10 of 12 HIV-1-infected children who started HAART at a median age of 1.9 months and maintained suppression for up to 5.5 years.

Van Zyl et al. (2015) have shown that children treated early had lower concentrations of HIV-1-infected cells compared to children treated later. A study done by Luzuriaga et al. (2014) also showed that plasma HIV-1 RNA was consistently undetectable in four perinatally HIV-1-infected youth (median age of 16.9) who received treatment early, very low amounts of HIV-1 DNA were detected and replication-competent virus was detected for only one out of the four early treated youth. Luzuriaga et al. (2014) also looked at four perinatally HIV-1-infected youth who received treatment late and was able to recover infectious virus from all four of these patients (although these patients were classified as young adults at the time of this study, their median age was 22.5 years). Rainwater-Lovett et al. (2017) also showed that the latent reservoir in 11 early treated, long-term suppressed perinatally infected children and adolescents was not inducible by qVOA and it was dominated by non-induced proviral genomes.

Therefore, taken together early cART can reduce HIV-1 replication, preserve the immune system, reduce HIV-1 replication-competent reservoirs and HIV-1 quasispecies diversity which provides a good basis towards achieving a cure.

1.6 Progress Towards an HIV-1 Cure

As discussed before, the latent reservoir that contains HIV-1 replication-competent virus hinders the ability to develop a functional or sterilising cure. A functional cure refers to silencing the latent reservoir through sustained virological control off ART (this is also known as remission) and a sterilising cure refers to completely eliminating the latent reservoir (Thomas et al., 2020).

1.6.1 Control of viral rebound (functional cures)

Functional cures are defined as the control of viral replication despite the presence of replication-competent HIV. Up until today, there have been a few reports of cases of functional cures where there has been sustained virological control off ART which is known as remission. The ‘Mississippi baby’ who had been initiated on triple ARV therapy close to the time of birth (30 hours after birth) and discontinued after 18 months of treatment is an example of a functional cure. There was no evidence of viral rebound, plasma HIV-1 RNA and proviral HIV-1 DNA were only intermittently detected at levels just above the limits of detection of the assays, and no HIV-1 antibodies were detected (Persaud et al., 2013). As they were not completely convinced of cure and due to some uncertain detection of low-level HIV-1 DNA signal, during the period of apparent remission (using ddPCR), Persaud et al. (2013) most likely chose to describe this case a “functional cure”. Regrettably, two years after treatment was stopped, remission ended, as HIV-1 RNA was detectable at 16 750 copies/mL. It is unlikely that the delayed rebound constituted a functional cure as there was no evidence of adaptive immune responses prior to the rebound and virus rebound occurred fast with accompanied CD4+ decline (Luzuriaga et al., 2015). Moreover, modelling suggests that the delay in rebound could have been due a very low reservoir size rather than control of viral replication (Hill et al., 2016).

It was also reported that a French girl who had been initiated on ART at three months of age, stopped ART sometime between five and seven years of age and maintained HIV-1 virological remission for over 12 years (Frange et al., 2016). Violari et al. (2019) recently

reported a South African child who initiated ART at 61 days old, followed by interruption when the child was 50 weeks old that was able to maintain post-treatment immune control for 9.5 years with very low, but detectable HIV-1 RNA and DNA levels.

Therapeutic vaccination initiates the host immune response to HIV-1 which results in elimination of rebound viraemia instead of directly targeting the sanctuary sites that host latently infected cells. In therapeutic vaccine trials, vaccine efficacy can be assessed by measuring the size of the latent reservoir, time to viral rebound and assessing the profile of the host immune response as the vaccine is given during sustained ART followed by a period of ART interruption. However, no study has yet been successful in achieving sustained viral remission in vaccinated patients. Davenport et al. (2019) showed that even with vaccines that block 80% of virus reactivations, after five weeks of ART interruptions plasma viraemia would rebound, suggesting that combinations of cure strategies may be required to increase effectiveness.

1.6.2 Strategies to reduce or eliminate viral reservoirs

At the present time, there have been two people cured of HIV-1-infection, the Berlin and the London patients. Both patients received allogenic stem cell transplantations from CCR5+ delta32/delta32 donors. The Berlin patient has tested negative for viral rebound for over 10 years and the London patient for two years without taking ARVs (Hütter et al., 2009) (Gupta et al., 2019). The infected cell pool was significantly depleted during pre-transplant conditioning and was replaced with donor cells that were resistant to infection with R5-tropic HIV-1, because of a large deletion in the CCR5+ co-receptor. This type of cure is not feasible for extensive use, because of CCR5+ delta32/delta32 donors being scarce and the fact that these cases were unique circumstances.

The “shock and kill” method (Figure 1.2) is one of the best-known approaches to cure HIV-1. It exposes the latently infected cells to immune clearance or viral CPE via utilising LRAs to induce viral gene expression in latently infected cells with the aim of reducing the size of the latent reservoir and limiting viral rebound (Deeks, 2012). A key challenge in using this approach is to achieve broad and efficient latency reversal without producing toxic side effects or global immune activation. Early studies that examined the use of LRAs used interleukin-2 (IL-2) to induce HIV-1 activation and found that a toxic response was produced and even when the dose of IL-2 was lowered, it was not able to adequately reduce the size

of the latent reservoir (Prins et al., 1999) (Lafeuillade et al., 2001). Novel LRAs work by altering the chromatin structure of the integrated provirus or by activating cellular transcription factors to induce HIV-1 gene expression. Abner and Jordan (2019) listed published LRAs and categorised them into six groups according to their mode of action: nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) stimulators, extracellular stimulators, toll like receptor (TLR) agonists, histone post-translational modification modulators, non-histone chromatin modulators and a mixed category of unique cellular mechanisms. Histone deacetylate inhibitors (HDACi) and histone methyltransferases inhibitors (HMTi) are examples of two leading LRAs in ongoing clinical trials. These two LRAs work by reversing epigenetic silencing which induces HIV-1 expression silencing (Archin et al., 2012) (Abner & Jordan, 2019). As mentioned earlier, one of the biggest challenges with the “shock and kill” method is to achieve global reactivation of HIV-1 from latently infected cells without it having a toxic effect. Another challenge, is to efficiently kill the latently infected cells. This happens either by viral CPE or by CTL mediated immune clearance. There have been reports of studies that successfully accomplished latency reversal in vivo, although they were not able to reduce the latent reservoir or increase time to viral rebound which indicated that all of the infected cells were not cleared (Archin, et al., 2014a) (Archin, et al., 2014b) (Elliott et al., 2015). Chomont et al. (2018) have indicated that long-term suppressed patients suffer from a loss of HIV-1 specific CTL responses, which may contribute to the loss of the “kill” response. This highlights the importance of finding a potent “kill” function, such as LRAs that are able to clear the infected cells efficiently or by combining treatment strategies to support CTL function. Therefore, TLR agonists offer potential since they are able to induce a broad anti-viral response and at the same time activate virus production followed by initiating immune clearance of HIV-1-infected cells (Tsai et al., 2017) (Macedo et al., 2018).

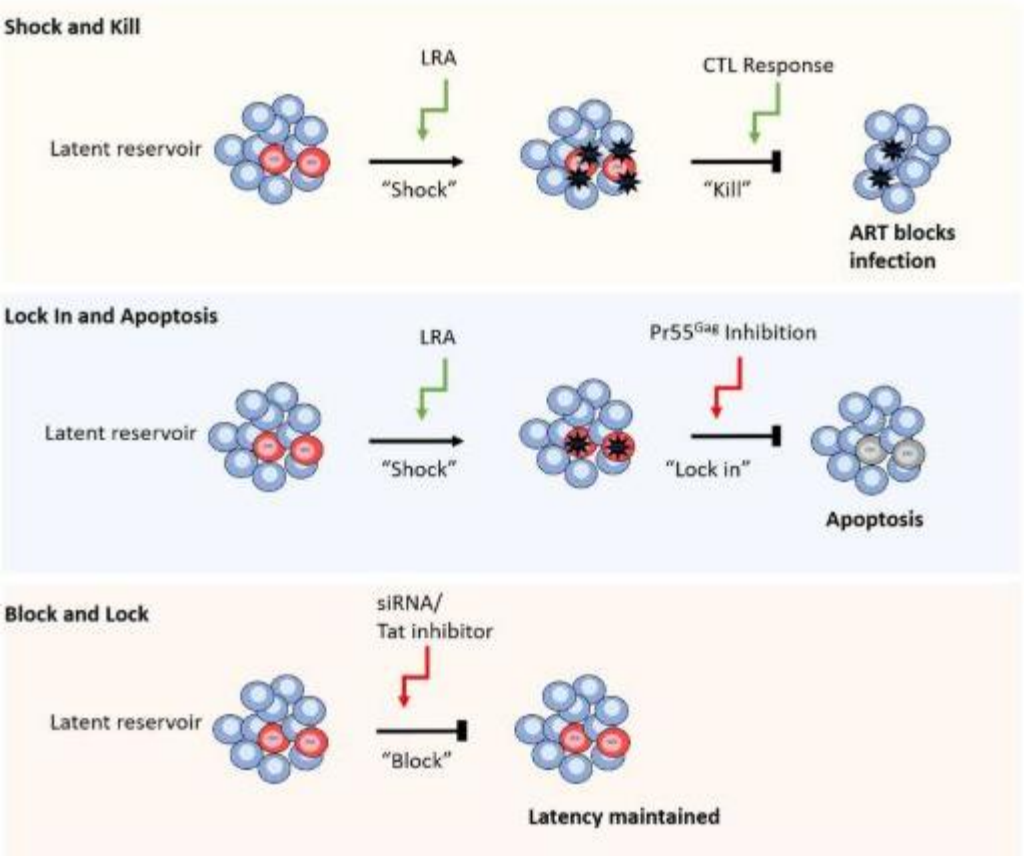

Figure 1.2: Different strategies for HIV-1 cure

HIV-1 latently infected cells are represented in red. (siRNAs: small interfering RNAs). Picture sourced from Thomas et al. (2020). Copyright © 2020 Thomas, Ruggiero, Paxton and Pollakis. This was an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY) (https://creativecommons.org/about/cclicenses/).

Tateishi et al. (2017) have found an alternate approach to avoid the need for CTL mediated cell clearance by blocking the release of virions which results in apoptosis of the infected cells known as the “lock in and apoptosis” method (Figure 1.2). This is achieved by blocking virus budding, causing viral particles to build-up resulting in apoptosis of the infected cells by using a novel compound that inhibits HIV-1 Pr55Gag.

The “block and lock” approach (Figure 1.2) is a novel cure strategy that has been proposed recently. This approach aims to reinforce latency so that viral rebound will be prevented when ART is interrupted (Mousseau et al., 2015). Transcriptional gene silencing (TGS) is induced through disruption of the chromatin structure which preserves the epigenetic mechanisms that maintain HIV-1 latency by using siRNAs (Méndez et al., 2018). Another way of enforcing HIV-1 latency is by inhibiting the HIV-1 positive regulator, Tat, which will

"lock" the replication cycle at the transcription step (Mousseau et al., 2015). The development of these approaches are still in pre-clinical stages and not yet in human trials.

Gene editing tools such as CRISPR-Cas9 and zinc-finger nucleases (ZFN) have greatly contributed to the hope of finding a cure for HIV-1. These tools target a variety of host or viral genes in order to induce resistance in the host through deleting integrated proviruses or enforcing viral latency. A clinical trial has evaluated the potential of ZFN in editing host CCR5+ resulting in partially genetic resistance to HIV-1 (Tebas et al., 2014). Wang *et al*. (2014) and Xu et al. (2017) have shown how CRIPSR-Cas9 can be used to edit the genes of CCR5+ and CXCR4+ which resulted in the host's cells being resistant to HIV-1. Dash et al. (2019) has also shown how CRISPR-Cas9 in combination with a drug delivery system can directly edit the proviral DNA and could therefore eliminate HIV-1-infection in mouse models. Even though this technology could practicably be used to target countless steps in the HIV-1 replication cycle it has a limitation in its delivery method since it requires lipid compounds or viral vectors (Xiao et al., 2019). Also, when considering the vast range of anatomical sanctuary sites which host a substantial proportion of latently infected cells, to achieve a cure with this technology is a major challenge since most of the latent reservoir will need to be targeted.

1.7 Implementing a Simple VOA in a South African Laboratory Setting

For this study's purposes, a simple yet sensitive VOA was implemented in the hope to recover infectious HIV-1 from children undergoing suppressive ART. To do this in a South African laboratory setting it was decided to simplify the assay by using MOLT-4 CCR5+ cells as the target cells for HIV-1 expansion instead of CD8+ depleted donor PBMCs as the preparation of CD8+-depleted lymphoblasts are labour intensive and costly, as mentioned before. The MOLT-4 CCR5+ cell line is a continuously growing cell line that grows in suspension and expresses high levels of CD4+, as well as the co-receptors CCR5+ and CXCR4+. The cell line was derived from MOLT-4 cells, that expressed high levels of CD4+ and CXCR4+, and it was engineered to express CCR5+ by transfecting a CCR5+ expressing plasmid into MOLT-4 cells. Therefore, this cell line is capable of supporting replication of X4-tropic and R5-tropic variants of HIV-1 (Baba et al., 2000).

A summary of the conventional qVOA versus the simplified MOLT-4 CCR5+ utilising qVOA can be seen in Figure 1.3. The VOA for this study was simplified even further by isolating total CD4+ T cells from HIV-1-infected patient PBMCs instead of resting CD4+ T cells. Also, since children would be assayed for replication-competent virus from latently infected cells and since ethically only a very small amount of blood was allowed to be drawn from children (only up to 10% of the total blood volume) (Howie, 2011), the total CD4+ T cells isolated from children would be plated only in replicates of 1 x 10^6 cells/well instead of in serial dilutions. Therefore, the frequency of the latently infected cells, reported in infectious units per million cells (IUPM), were not determined, as the cells were not plated in limiting dilutions. For this study's purposes, an in-house RNA RT-qPCR assay was used to determine whether there was outgrowth of virus over time, as this assay measured cell-free virus, positive results were confirmed with the p24 ELISA and the detection rate of the p24 ELISA was compared to that of the in-house RNA RT-qPCR assay to determine which assay detected infectious virus earlier.

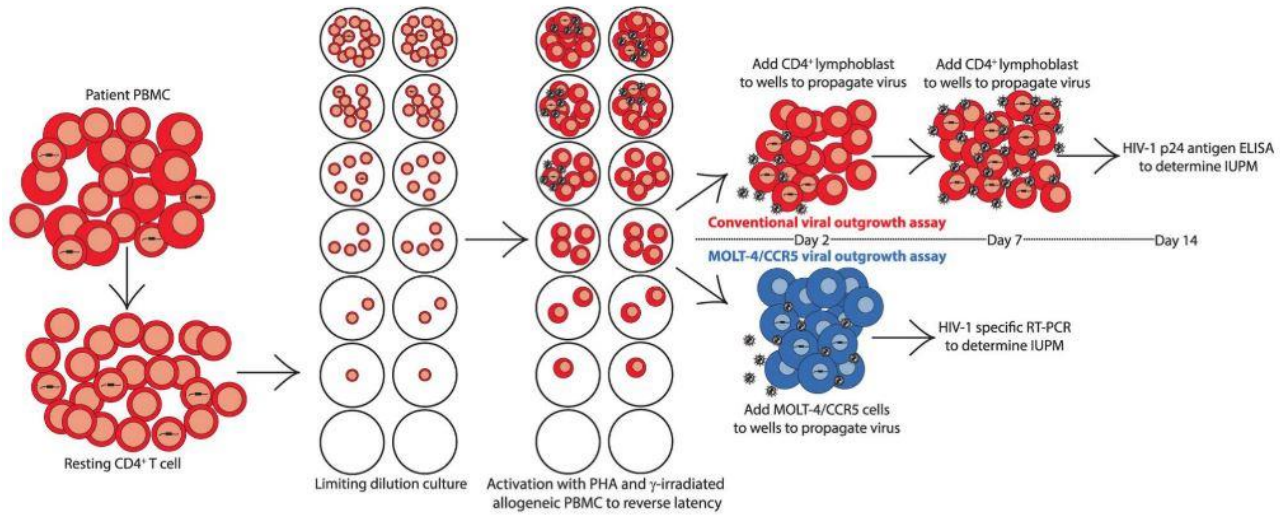

Figure 1.3: The Conventional VOA and the MOLT-4 CCR5+ VOA

(RT-PCR: reverse transcriptase polymerase chain reaction assay). Picture sourced from © 2013 Laird et al. This was an open-access article distributed under the terms of the Creative Commons Attribution License.

1.8 Rationale

1.8.1 Summary of literature review

Even though there have been promising results with the early initiation of cART in children as it is able to reduce HIV-1 plasma viral loads to being clinically undetectable, reduce

mortality rates among HIV-1-infected patients, increasing life expectancy and even to reduce the reservoir size, it is not successful in eliminating the dormant replication-competent HIV-1 that persist in latently infected cells from early infection. This results in life-long cART being necessary to reduce the chances of viral rebound which comes with side effects and other challenges such as adherence, availability in resource limited settings and the possible accumulation of drug resistance mutations with long term use. For this reason, research continues to find a cure for HIV-1, either by targeting HIV-1 reservoirs directly (eradication cure approach) or by controlling viral rebound from reservoirs (functional cure approach).

Assays to study the latent reservoir contribute greatly in understanding HIV-1 persistence, which ultimately contributes to the advancement of an HIV-1 cure. For this reason, a VOA was developed to study HIV-1 persistence in children on suppressive cART, since the VOA is the only assay that can measure the true HIV-1 reservoir as it measures replication-competence and thereby excludes defective viruses.

1.8.2 Research question

This study aims to address the following research question:

Are viruses released from infected cells in children on suppressive ART able to infect new MOLT-4 CCR5+ cells, replicate and release new infectious HIV-1 virions, referred to as viral outgrowth (measured by the RNA RT-qPCR assay and confirmed with the p24 ELISA)?

1.8.3 Aim and objectives

Figure 1.4 gives an overview of the aim and objectives that have been set for this project to be able to answer the above research question.

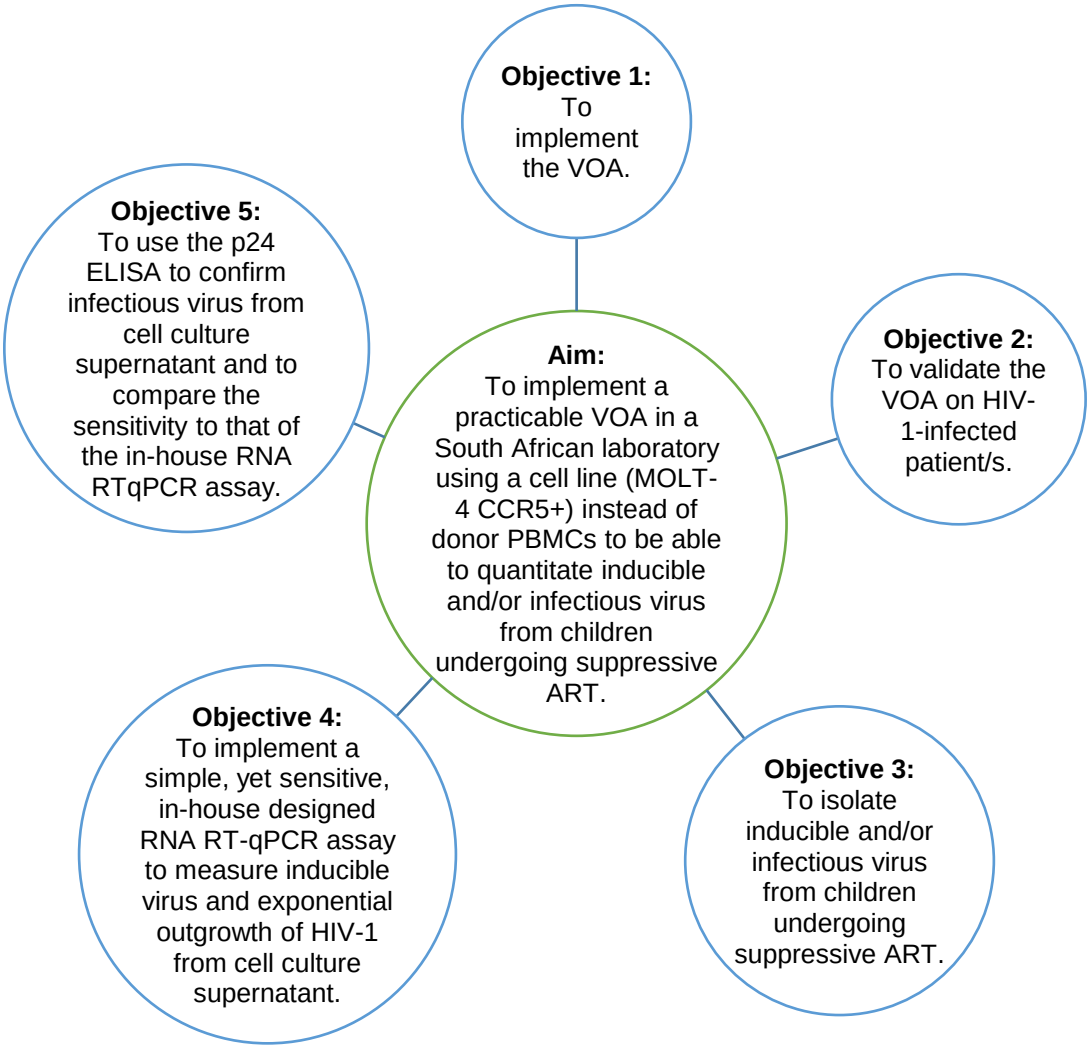

Figure 1.4: Overview of the aim and objectives for this research project

Chapter 2

Materials and Methods

2.1 Ethical Approval

This study was approved on the 27th of March 2018 by the Health Research Ethics Committee (HREC). Project identity (ID) number: 6211. Reference number: N18/02/020 and reference reservoir clinical study M14/07/029. These ethical approvals included the use of samples from HIV-1 positive individuals (including Post-Children with HIV Early Antiretroviral Therapy randomised trial [CHER] patients), and anonymised material for assay optimisation.

2.2 Study Specific Definitions

2.2.1 Inducible HIV-1

Inducible HIV-1 in this study specifically refers to virus that gets released from infected cells (after stimulation) but does not necessarily result in new rounds of infection as evident from exponential viral growth kinetics in permissive cells such as MOLT-4 CCR5+ cells (this can be measured by a RT-qPCR assay, such as the in-house implemented RNA RT-qPCR assay, from extracted VOA cell culture supernatant HIV-1 RNA).

2.2.2 Viral outgrowth

Viral outgrowth in this study refers to viruses that get released from infected cells in children on suppressive ART that is able to infect new MOLT-4 CCR5+ cells, replicate and release new infectious HIV-1 virions, which can be observed from an exponential increase in HIV-1 RNA with the in-house RNA RT-qPCR assay and high titres of infectious virus can be confirmed with the p24 ELISA from VOA cell culture supernatants.

2.2.3 Integrase cell associated total HIV-1 DNA quantitative assay value

For this study, patients were selected that had an integrase cell associated total HIV-1 DNA quantitative assay (iCAD) value of >50 copies per million cell equivalents in order to increase the chances of recovering infectious HIV-1 from these children. The iCAD assay is a qPCR

assay that targets the p31 region of *pol* (*integrase*) and quantifies total HIV-1 DNA in infected T cells. Although this iCAD cut off value that was used to select the patients might seem low, it was relatively high for these children because the children were treated early with ART.

2.2.4 HIV-1 DNA diversity

The diversity of HIV-1 cell associated DNA was calculated according to the cell associated DNA-single genome sequencing (CAD-SGS) assay. This assay is a highly sensitive method for PCR amplification and sequencing where DNA is diluted to an end-point where by Poisson statistics, 30% of all replicates should have amplification as a result of a single genome. For this study's purposes, a diversity of >1.5% was seen as high. The diversity was calculated for one of the patient samples assayed with the VOA.

2.2.5 ART suppressed patients

In this study, ART suppressed patients refer to patients that had a HIV-1 RNA viral load of <400 copies/mL.

2.2.6 Viraemic patient samples

A viraemic patient in this study refers to a patient that had a HIV-1 RNA viral load of >400 copies/mL.

2.3 Study Design and Study Population

This descriptive study aimed to characterise HIV-1 latency in children on long-term suppressive therapy. Figure 2.1 is a schematic overview of the overall study design followed to achieve this study's aim and objectives.

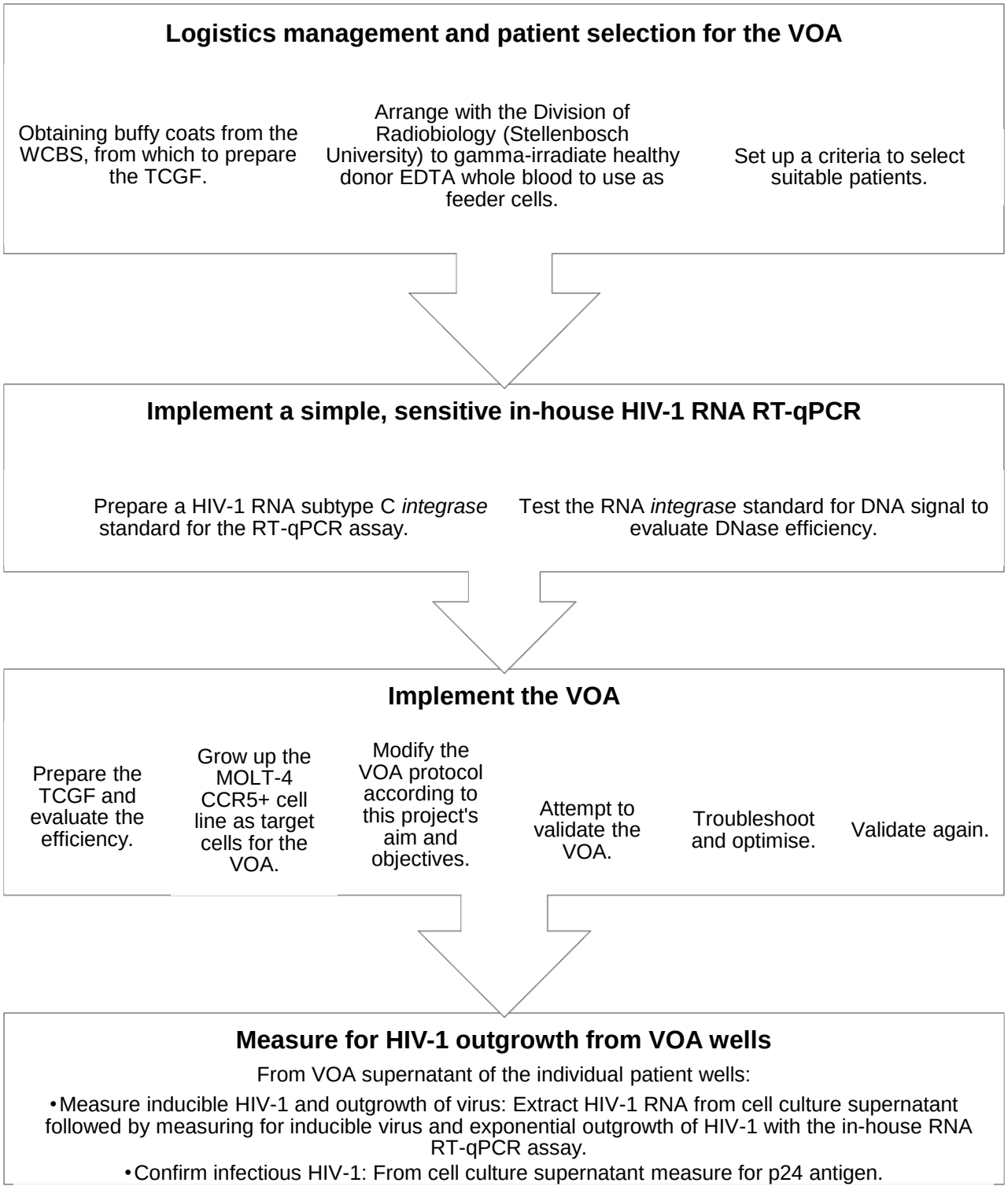

Figure 2.1: Schematic overview of the overall study design

(WCBS: Western Cape Blood Service. TCGF: T cell growth factor. EDTA: Ethylenediaminetetraacetic acid).

Cohorts studied and participant selection criteria

The study participants for this investigation included the follow-up of children who were recruited as part of the CHER cohort between 2005 and 2011 known as the Post-CHER cohort.

The CHER study was conducted in South Africa in Johannesburg at the HIV Research Unit, Chris Hani Baragwanath and in Cape Town at the Children's Infectious Diseases Clinical Research Unit, Tygerberg Hospital. Infants were recruited into the study based on the following criteria: confirmation of HIV infection by a positive PCR test for HIV-1 DNA and a plasma HIV-1 RNA PCR result of >1000 copies/mL. Infants were assigned into two trial parts based on their percentage of CD4+ T cells: those with a CD4+ T cell percentage of 25% or more were assigned to the main trial (Part A) and infants with a CD4+ T cell percentage of less than 25% formed part of Part B. Part B participants were excluded from primary analysis where they compared immediate to delayed therapy. Participants from Part A were randomised into three arms and those from Part B were randomised into two arms. In Arm one, the patients were given delayed ART therapy (Part B participants were excluded from this arm). In Arm two, therapy was received for 40 weeks and then subsequently interrupted. In Arm three, the infants received therapy for 96 weeks followed by subsequent interruption. If the CD4+ T cell percentage was <20% (in the case of infants younger than 12 months, this was a CD4+ T cell percentage of <25%) therapy would be initiated for participants in Arm one (deferred therapy arm) or re-initiated for participants in Arm two or three. Participants started on a first line ART regimen which consisted of: zidovudine (AZT) and lamivudine (3TC) with lopinavir-ritonavir (LPV/r). The second line regimen was didanosine (DDI), abacavir (ABC), and efavirenz (EFV) or nevirapine (NVP) (Violari et al., 2008) (Cotton et al., 2013).

A subgroup of the children was retained to be part of the post-CHER descriptive study to investigate HIV-1 reservoirs and neurocognitive outcome (succeeding the conclusion of the CHER clinical trial). For this project's purposes, two children from the post-CHER descriptive study, were selected, general information on these patients can be seen in Table 2.1. These two Post-CHER children had to have an iCAD value of >50 copies per million cell equivalents, the viral load at the time point chosen had to be suppressed, but they also had to be recently viraemic (the viral load of the prior visit [V] to the one chosen had to be

unsuppressed). The iCAD assay was not performed routinely, it was an in-house assay that was performed by a research team member and by the author of this thesis. These two children were selected to assay for replication-competent HIV-1 with the VOA as it was thought that children with a recent episode of viraemia as well as high numbers of total HIV-1 DNA would increase the chances of recovering infectious virus. Since it has been shown in literature that it is difficult to recover infectious virus from early treated children.

Table 2.1: General information on the two Post-CHER patients selected for the VOA

Post-CHER patient ID number	337756	341622
Gender	Female	Male
Date of birth	17/08/2006	24/12/2007
ART initiation date	26/10/2006	26/02/2008
Age at therapy initiation	2 months (64 days)	2 months (64 days)
CHER study arm	Part A, Arm 2	Part A, Arm 2
Treatment strategy	Interrupted (368 days)	Interrupted (392 days)
ART treatment history:		
Date started (Regimen)	26/10/2006 (AZT/3TC/LPV/r)	26/02/2008 (AZT/3TC/LPV/r)
Date stopped	06/08/2007	02/12/2008
Date of reinitiation (Regimen)	08/08/2008 (AZT/3TC/LPV/r)	12/01/2010 (AZT/3TC/LPV/r)

2.4 Design and Implementation of a Sensitive HIV-1 RNA RT-qPCR Assay to Measure Inducible HIV-1 and outgrowth of HIV-1

2.4.1 Preparation of the HIV-1 subtype C RNA *integrase* standard for the HIV-1 RNA RT-qPCR assay

2.4.1.1 Amplification procedure from a linearised subtype C infectious clone (pMJ4)

To implement a sensitive HIV-1 RNA RT-qPCR assay, an RNA HIV-1 standard had to be generated to normalise the quantity of virus detected. This was done by cloning the p31 region of *pol* (*integrase*) from plasmid MJ4 (12 833 base pairs [bp], Figure 2.2) that contained an infectious clone of HIV-1 subtype C. The pMJ4 clone was obtained from the National Institutes of Health (NIH) AIDS Reagent Program, Division of AIDS, National

Institute of Allergy and Infectious Diseases (NIAID), from Drs. Thumbi Ndung'u, Boris Renjifo and Max Essex (Ndung'u et al., 2001). Amplicons of 418 bp were amplified from the p31 region of *pol* (*integrase*) using a designed set of primers (see Addendum A1) targeting the *integrase* gene in the linearised 12 833 bp MJ4 plasmid (Ndung'u et al., 2001). The SimpliAmp Thermal Cycler (Applied Biosystems, Waltham, USA) was used for amplification, and the master mix had a total volume of 50 µL per reaction. A total of 10 reactions were performed to ensure that there would be enough DNA in the subsequent steps to generate the RNA standard. The master mix ingredients and the cycle conditions can be seen in Addendum A1. The end-point PCR products were stored at -20°C.

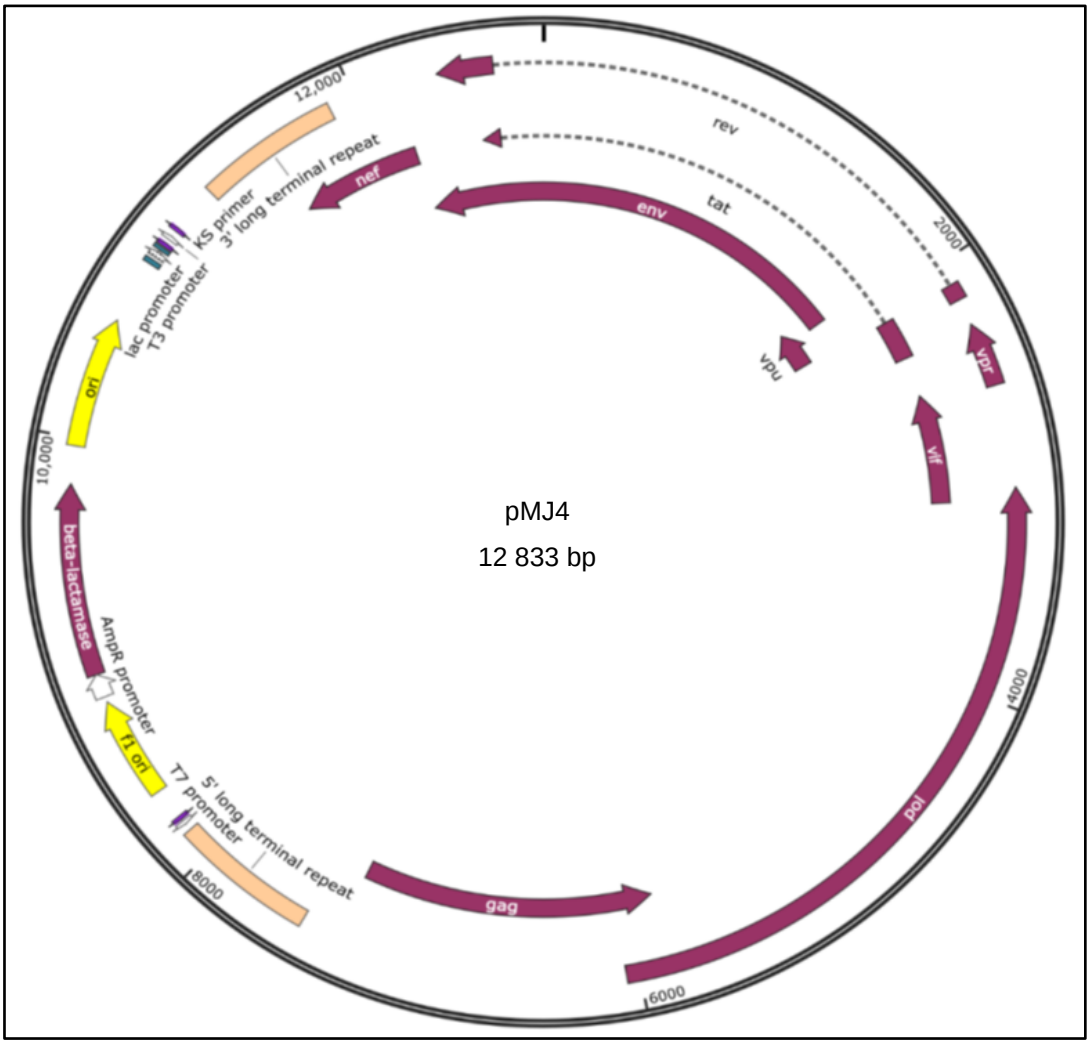

Figure 2.2: Map of pMJ4 (Ndung'u et al., 2001)

2.4.1.2 End-point PCR amplicon analysis and purification

Gel-electrophoresis, consisting of 1% agarose (Lonza, Basel, Switzerland) and 1X SB buffer were used to determine the DNA band sizes of the end-point PCR amplicons. The gel was examined under ultraviolet (UV) illumination to ensure that the DNA bands were 418 bp. The 1X SB buffer was prepared by first making 20X SB buffer, by adding 8 g Sodium Hydroxide (Merck, Darmstadt, Germany) and 47 g Boric Acid (Merck, Darmstadt, Germany) to1 L Milli-Q water (Merck, Darmstadt, Germany). Then 50 mL of 20X SB buffer was added to 950 mL Milli-Q water (Merck) to make 1X SB buffer (Refer to Addendum A2). To determine the band sizes, a 100 bp DNA ladder (GeneDireX, Inc., Taoyuan, Taiwan) was used. All 10 of the PCR products, together with the 100 bp DNA ladder (GeneDireX, Inc), including two wells on the gel that contained no template controls (NTCs) (nuclease-free water [Qiagen, Venlo, Netherlands]) were mixed with Novel Juice (GeneDireX, Inc., Taoyuan, Taiwan) as loading dye and added onto the gel. Voltage was set at 90 V on the ENDURO™ Gel XL Electrophoresis System (Labnet International Inc., Edison, USA). The end-point PCR amplicons were run for 45 minutes. The gel was examined using the UV-ITEC Prochem Gel Dock System (Whitehead Scientific Pty Ltd, Cape Town, SA).

To purify the DNA amplicons, another 1% agarose (Lonza) gel, dissolved with EZ-Vision Bluelight DNA staining dye (VWR International, Radnor, USA) at a final concentration of 1X, had to be run. All 10 DNA amplicons, two NTCs (nuclease-free water [Qiagen]) and two 1 kilobases (kb) DNA ladders (GeneDireX, Inc., Taoyuan, Taiwan) had to be run on the gel. Blue/orange 6X Dye (Promega Corp., Madison, USA) was used as loading dye. The 1 kb DNA ladders (GeneDireX, Inc.) were loaded on each side of the gel for increased accuracy in reading of the sizes of the 10 DNA amplicons. The 10 DNA amplicons were gel extracted, using the manufacturer's instructions from the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany). The gel extracted samples were then pooled into four different aliquots (Aliquot one: tubes one, two and three. Aliquot two: tubes four, five and six. Aliquot three: tubes seven and eight. Aliquot four: tubes nine and 10). The aliquot that obtained the better purity according to the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA) was used in subsequent experiments. Purity can be measured in two ways by the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). The main measure of purity is the absorbance at 260 nm and 280 nm which should be ~1.8 for DNA and ~2.0 for RNA (demonstrated by the 260/280 ratio). The secondary measure of

nucleic acid purity is the 260/230 ratio which demonstrates the absorption at 260 nm and 230 nm, and it ranges between 1.8-2.2 for DNA and RNA (Thermo Fisher Scientific, 2010).

2.4.1.3 Cloning of the purified 418 bp amplicon into vector pTZ57R/T

The resulting gel-extracted amplicon was cloned into vector pTZ57R/T (Figure 2.3) using the InsTAclone™ PCR Cloning kit (Thermo Fisher Scientific, Waltham, USA), downstream of the T7 promotor. This kit makes use of the terminal transferase activity of *Taq* DNA polymerase and other non-proofreading thermostable DNA polymerases. Enzymes like these work by adding a single 3'-Adenine (A) overhang to both ends of the PCR product. This enables direct cloning into a linearised cloning vector with single 3'-dideoxy Thymine (ddT) overhangs. These overhangs at the vector cloning site not only make cloning possible, but also prevents the recirculation of the vector. Therefore, more than 90% of recombinant clones would contain the vector with an insert. Recombinant clones are selected based on blue/white screening (white colonies represent the vector with the insert). Figure 2.4 shows a summary of the cloning procedure.

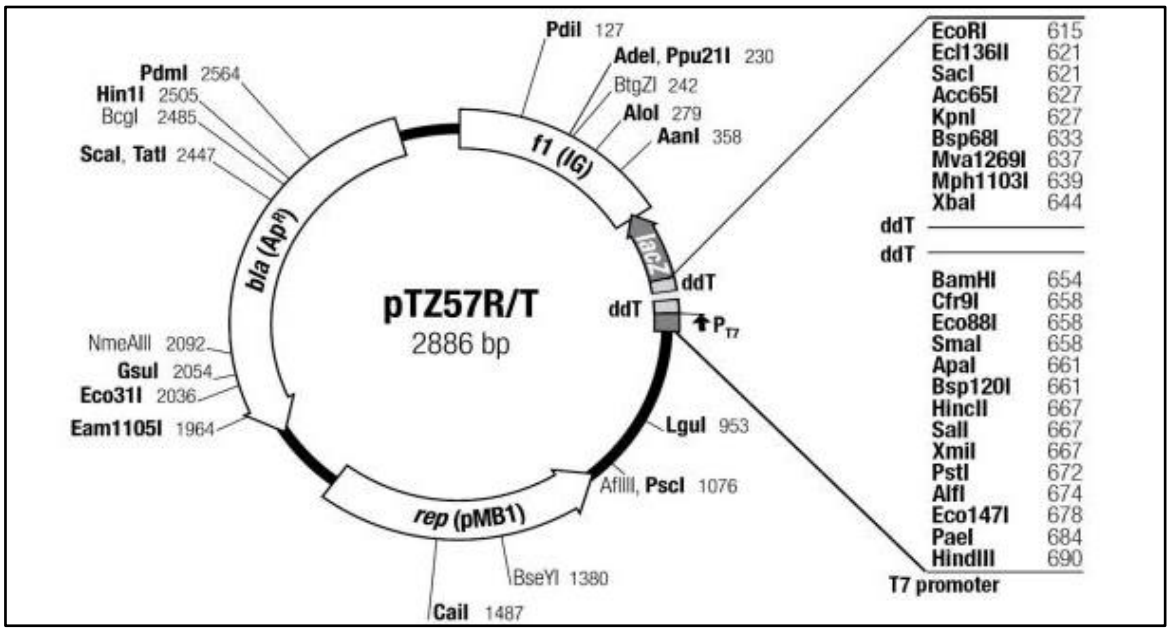

Figure 2.3: Map of vector pTZ57R/T (InsTAclone™ PCR Cloning kit, Thermo Fisher Scientific)

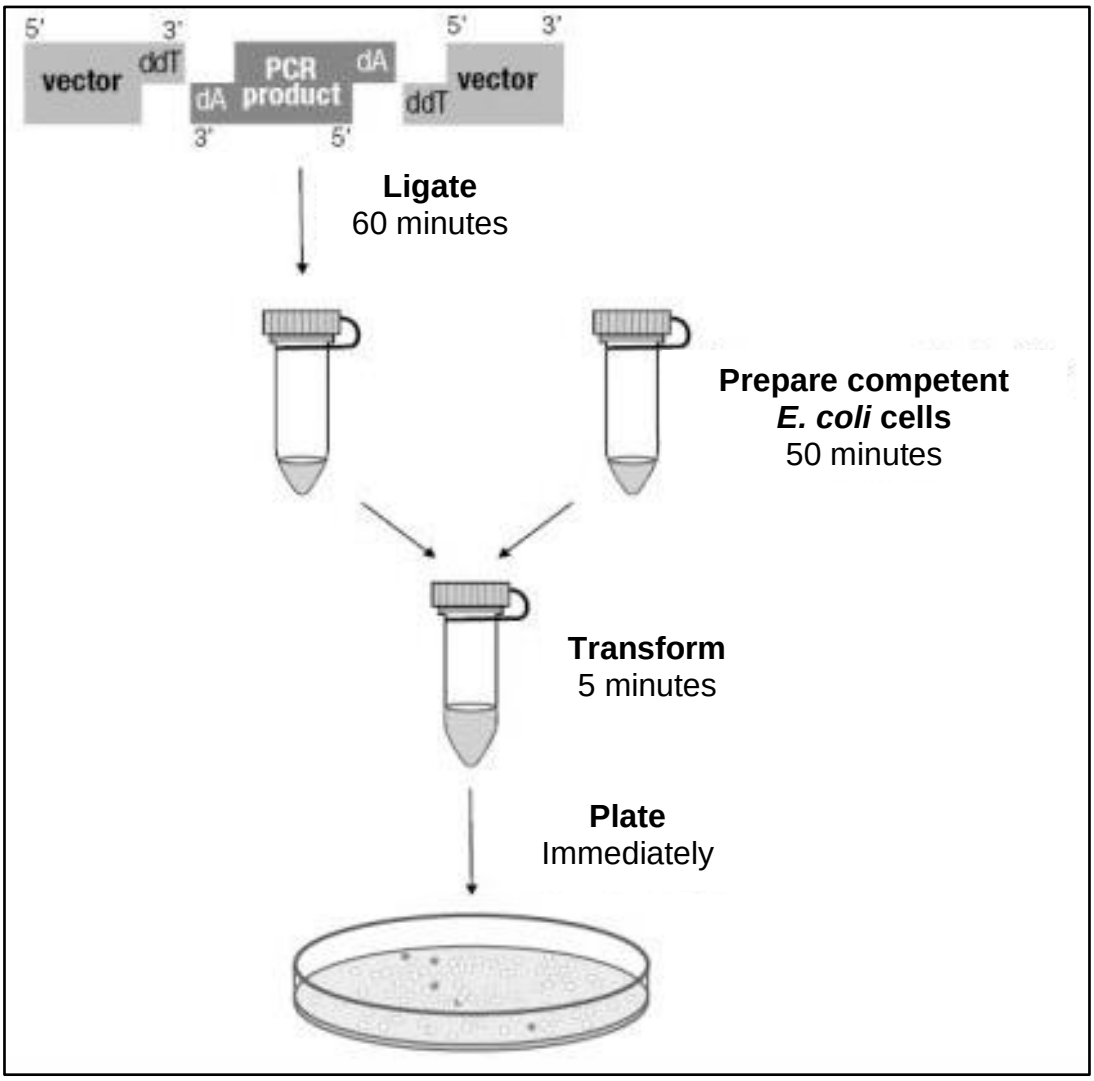

Figure 2.4: Summary of the cloning procedure using the InsTAclone™ PCR Cloning kit (Thermo Fisher Scientific)

The first step of the cloning procedure involved ligating the 418 bp amplicon into the pTZ57R/T vector. For this step the manufacturer's instructions were followed with some alterations to the incubation step (conditions for the incubation step can be seen in Addendum A3). A positive control PCR fragment of 0.52 pmol ends (InsTAclone™ PCR Cloning kit [Thermo Fisher Scientific]) and a negative control were included in the ligation reaction for quality control purposes.

The second step involved the transformation of plasmid pTZ57R/T with the 418 bp insert into One Shot™ TOP10 Chemically Competent *Escherichia coli* (*E. coli*) cells (Thermo Fisher Scientific, Waltham, USA). The following protocol was followed: selective Luria Broth (LB) agar medium petri dishes (Thermo Fisher Scientific, Waltham, USA) were warmed up

at 37°C for 30 minutes. The LB agar medium was made as follows: combine 10 g LB broth (Sigma-Aldrich, St. Louis, USA) and 7.5 g LB agar (Sigma-Aldrich, St. Louis, USA), and top it up with Milli-Q water (Merck) up to 500 mL. This was autoclaved for 30 minutes. After it cooled down completely, ampicillin (Tocris Bioscience, Bristol, UK) was added at a final concentration of 0.05 mg/mL. The LB agar medium plates were poured and allowed to solidify. The plates were stored upside down at 4°C until used (refer to Addendum A4 for the LB agar medium recipe). Meanwhile, a heat block was equilibrated to 42°C, a vial of Super Optimal broth with Catabolite repression (SOC) Medium (Condalab, Madrid, Spain) was warmed up to 37°C and a master mix for each petri dish (Thermo Fisher Scientific) was prepared containing: 50 µg/mL 5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside (X-Gal) ready-to-use solution (Thermo Fisher Scientific, Waltham, USA), 20 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) ready-to-use solution (Thermo Fisher Scientific, Waltham, USA), and 100 µg/mL of ampicillin (Tocris Bioscience). This master mix was spread onto the petri dishes followed by incubation at 37°C for 30 minutes. After this, a vial of One Shot™ TOP10 Chemically Competent *E. coli* cells (Thermo Fisher Scientific) for each transformation reaction was thawed on ice. For this step 2.5 µL of the ligation positive and negative controls, 1.25 µL of the transformation negative control (nuclease-free water [Qiagen]), and 1.25 µL of the positive pUC19 Control DNA at a concentration of 10 pg/µL (Thermo Fisher Scientific, Waltham, USA) were added to separate vials of One Shot™ TOP10 Chemically Competent *E. coli* cells (Thermo Fisher Scientific) and mixed gently by tapping the vial. The vials were placed on ice for 30 minutes. The cells were then heat-shocked to allow the *E. coli* cells (Thermo Fisher Scientific) to take up the plasmid vector by placing the vials in a heat block at 42°C for 30 seconds and then on ice for 2 minutes. A volume of 250 µL 37°C pre-warmed SOC Medium (Condalab) was added to each vial aseptically. The vials were capped tightly and shaken horizontally at 37°C for 1 hour at 225 rotations per minute (rpm) in a Labcon shaking incubator 3081U (Labcon, Petaluma, USA). A volume of 200 µL from each transformation reaction was spread on 37°C pre-warmed selective LB agar medium petri dishes. The petri dishes were inverted and incubated overnight at 37°C

After the transformation step and allowing the bacteria to grow overnight on selective LB agar medium petri dishes at 37°C, a technique known as blue/white colony screening was used to select colonies that contained the vector with the recombinant DNA (white colonies).

Five big white colonies were picked for bacterial culture. Three out of the five colonies were combined and incubated at 37°C in a Labcon shaking incubator 3081U (Labcon) (225 rpm) in a 15 mL polypropylene nonpyrogenic sterile conical tube (Lasec® Group, Cape Town, SA), containing 5 mL LB broth solution, for 6-8 hours and then transferred to a 250 mL Erlenmeyer flask (Lasec® Group, Cape Town, SA) to scale up the culture. The LB broth solution was prepared by adding Milli-Q water (Merck) up to 500 mL to 10 g LB broth (Sigma-Aldrich). The LB broth solution was autoclaved for 30 minutes followed by adding ampicillin (Tocris Bioscience) to a final concentration of 0.05 mg/mL after the broth had cooled down completely. The broth was stored at 4°C until used (Addendum A4). The culture was scaled up by diluting the starter culture from 1:1000 to 1:10 000 by adding the 5 mL cultured medium to 45 mL fresh LB broth solution. The two other white colonies were also added to two 15 mL conical tubes (Lasec® Group) containing 5 mL LB broth solution. These two 15 mL conical tubes (Lasec® Group) together with the scaled-up culture in the 250 mL Erlenmeyer flask (Lasec® Group) were then cultured overnight (12-16 hours) at 37°C in a Labcon shaking incubator 3081U (Labcon), shaking at 225 rpm. The scaled-up culture (250 mL Erlenmeyer flask [Lasec® Group]) was used in downstream experiments to generate the RNA standard while the two 15 mL conical tubes (Lasec® Group) that contained the smaller culture volumes were used to make glycerol stocks. Glycerol stocks of these two aliquots were prepared by taking 500 µL of the overnight culture from each aliquot and adding it to 500 µL of 50% glycerol (100% glycerol [Sigma-Aldrich, St. Louis, USA] diluted with Milli-Q water [Merck]). As many aliquots of bacterial glycerol stocks were made and stored at -80°C for future use.

2.4.1.4 Purification of the plasmid DNA followed by sequencing

Plasmid DNA from the scaled-up culture in the 250 mL Erlenmeyer flask (Lasec® Group) was isolated with the Thermo Scientific™ GeneJET™ Plasmid Midiprep Kit (Thermo Fisher Scientific, Waltham, USA). This kit isolates high quality plasmid DNA from recombinant *E. coli* cultures on a large-scale. The manufacturer's instructions were followed to purify plasmid DNA. At the end of this purification protocol there were three aliquots of plasmid DNA resuspended in 300 µL elution buffer (Thermo Scientific™ GeneJET™ Plasmid Midiprep Kit [Thermo Fisher Scientific]) each. The aliquot that obtained better DNA purity according to the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific) was used for downstream experiments.

The pure plasmid DNA was sequenced to ensure that the 418 bp DNA amplicon was inserted into the vector and that it was indeed HIV-1 DNA from the *integrase* gene from the MJ4 plasmid. For sequencing purposes, the sample had to be at a concentration of between 15 and 25 ng/µL. A set of M13/pUC sequencing primers (Thermo Fisher Scientific, Waltham, USA [Addendum A5]) at a final concentration of 5 pmol/µL each were used for sequencing. The sequencing master mix and the cycle conditions for the sequencing reaction can be seen in Addendum A5. The SimpliAmp Thermal Cycler (Applied Biosystems) was used for the sequencing PCR reaction.

2.4.1.5 Linearisation of the plasmid DNA

After determining if the 418 bp DNA amplicon was inserted in the correct position with sequencing, the plasmid was linearised with the restriction enzyme EcoRI (Promega Corp., Madison, USA) downstream of the T7 promotor. The restriction enzyme, EcoRI (Promega Corp.), cuts the plasmid once in the multiple cloning site (MCS) region at position 615 bp. For the linearisation reaction to be efficient, 0.2-1.5 µg plasmid DNA had to be added to the reaction (in this case 1.5 µg of plasmid DNA was added to the reaction). The linearisation master mix and the cycling parameters can be seen in Addendum A6. The SimpliAmp Thermal Cycler (Applied Biosystems) was used for the linearisation reaction.

2.4.1.6 Purification of the linearised plasmid and ethanol precipitation to concentrate the DNA

After linearisation, the product was purified further with the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel) following manufacturer's instructions. Four aliquots from the linearisation reaction were combined into one tube, making two aliquots each of 80 µL. At the end of this purification step, there were two aliquots of 30 µL each.

This purification step was followed by ethanol precipitation to concentrate the DNA (Oswald, 2007), to ensure a sufficient DNA concentration for *in vitro* transcription. For ethanol precipitation purposes, an in-house method was followed: 3 M Sodium Acetate (Thermo Fisher Scientific, Waltham, USA) at a tenth of the volume of DNA was added to the tubes that contained the DNA. Three times the amount of DNA of ice cold 100% ethanol (Sigma-Aldrich, St. Louis, USA) was also added to each tube. The solution was thoroughly mixed using a vortex mixer. Glycogen (Roche Holding AG, Basel, Switzerland) at a concentration

of 20 mg/mL was added to each tube after mixing. Each tube was then placed at -20°C to precipitate it overnight. The following day the tubes were centrifuged in a Prism™ microcentrifuge (Labnet International Inc., Edison, USA) at 17 000 relative centrifugal force (RCF) for 30 minutes. The pellets were then washed twice with 500 µL ice cold 75% ethanol (100% ethanol [Sigma-Aldrich] diluted with Milli-Q water [Merck]). After the second washing step, the 75% ethanol was aspirated off without touching the pellet and the tubes were centrifuged at 17 000 RCF for 10 seconds with the Prism™ microcentrifuge (Labnet International Inc.) to remove any residual ethanol. After removing the residual ethanol, the pellets were air dried. Once the pellets were dry, 10 µL of diethyl pyrocarbonate (DEPC)-treated water (component of the Thermo Scientific™ TranscriptAid™ T7 High Yield Transcription Kit [Thermo Fisher Scientific, Waltham, USA]) was added to each aliquot for resuspension. This was then thoroughly mixed using a vortex mixer. The samples' purity and concentration were determined using the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific) and the sample that had a greater purity and a higher DNA concentration was used for *in vitro* transcription.

2.4.1.7 *In vitro* RNA synthesis and treatment with DNase I

In vitro RNA synthesis was performed using the Thermo Scientific™ TranscriptAid™ T7 High Yield Transcription Kit (Thermo Fisher Scientific). Manufacturer's instructions were followed for performing *in vitro* transcription. An overview of the process can be seen in Figure 2.5. In summary, 1 µg of the purified, linearised plasmid template DNA in combination with a positive control provided with the kit (Control DNA at a concentration of 0.5 µg/µL), and a negative control (nuclease-free water [Qiagen]) were added to the *in vitro* RNA synthesis reaction and incubated at 37°C for two hours using the SimpliAmp Thermal Cycler (Applied Biosystems).

After *in vitro* transcription, the RNA was treated with DNase I (Thermo Scientific™ TranscriptAid™ T7 High Yield Transcription Kit [Thermo Fisher Scientific]) to remove any template DNA. The *in vitro* transcribed template RNA as well as the positive and negative control reactions were treated with DNase I (Thermo Fisher Scientific) by adding two units of DNase I (Thermo Fisher Scientific) for every 1 µg of DNA template. The RNA product, together with the positive and negative controls were incubated at 37°C using the SimpliAmp Thermal Cycler (Applied Biosystems) for 15 minutes.

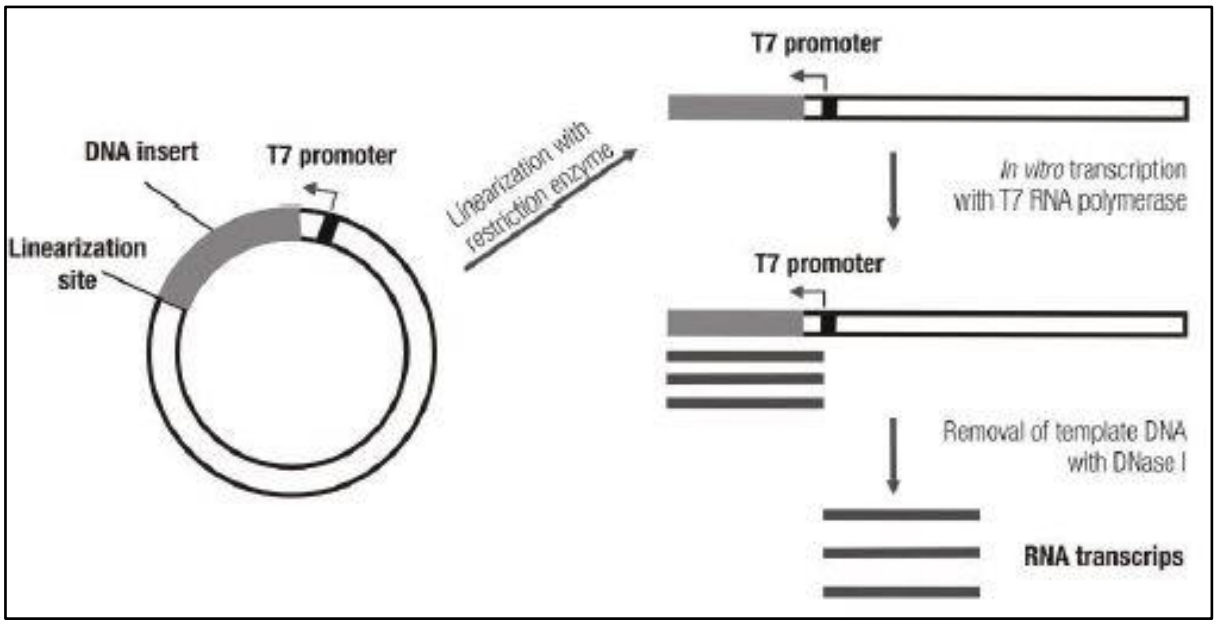

Figure 2.5: *In vitro* transcription from the linearised plasmid template

2.4.1.8 RNA purification and analysis

The RNA transcript in combination with the positive and negative controls were purified with the PureLink® RNA Mini Kit (Invitrogen, Waltham, USA) from any residual DNase I (Thermo Fisher Scientific) to use the RNA transcript downstream as an RNA HIV-1 standard in the HIV-1 RNA RT-qPCR assay. For purifying the RNA transcript and the positive and negative controls, the specific protocol “Purifying RNA from Liquid Samples/RNA Clean-Up” from the kit was followed. In summary, the samples were lysed and homogenized in the presence of guanidinium isothiocyanate (PureLink® RNA Mini Kit [Invitrogen]) which is a chaotropic salt capable of protecting the RNA from endogenous RNases. Then a 100% ethanol (Sigma-Aldrich) was added to the samples. Thereafter the samples were processed through spin cartridges (PureLink® RNA Mini Kit [Invitrogen]) that contained clear silica-based membranes to which the RNA would bind. Subsequent washing steps were used to get rid of any other impurities. The purified RNA was then eluted in RNase-free water (PureLink® RNA Mini Kit [Invitrogen]). Finally, the purity and concentration of the different samples (RNA transcript, positive and negative controls) were determined using the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific) to ensure that the samples were indeed purified from any residual template DNA, DNases and proteins that could interfere with downstream applications or could negatively affect the stability of the stored RNA and to

check that the RNA transcript was indeed RNA and not DNA. The purified RNA was stored at -80°C until future use.

A gel, that consisted of 1% agarose (Lonza) and 1X SB buffer, was used to evaluate the size of the RNA transcript and the positive control. Manufacturer's instructions from the Thermo Scientific™ TranscriptAid™ T7 High Yield Transcription Kit (Thermo Fisher Scientific) were followed to perform gel-electrophoresis. In summary, the positive and negative control reactions were prepared as follows: each control reaction was diluted 40-fold, then three microliters of each diluted control reaction was mixed with 3 µL of 2X RNA Loading Dye Solution (Thermo Scientific™ TranscriptAid™ T7 High Yield Transcription Kit [Thermo Fisher Scientific]). The samples were then incubated at 70°C for 10 minutes and chilled on ice prior to loading. The RNA transcript was diluted with DEPC-treated water (Thermo Scientific™ TranscriptAid™ T7 High Yield Transcription Kit [Thermo Fisher Scientific]) to a final concentration of 0.25 µg/µL. Four microliters of the diluted RNA transcript was mixed with 4 µL 2X RNA Loading Dye (Thermo Scientific™ TranscriptAid™ T7 High Yield Transcription Kit [Thermo Fisher Scientific]). The diluted RNA transcript and the RiboRuler RNA Ladder, High Range, ready-to-use (Thermo Scientific™ TranscriptAid™ T7 High Yield Transcription Kit [Thermo Fisher Scientific]) was incubated at 70°C for 10 minutes. The diluted RNA transcript and the ladder were chilled on ice for three minutes and briefly centrifuged before loading onto the gel. The gel was run for an hour at 65 V using the ENDURO™ Gel XL Electrophoresis System (Labnet International Inc.). The UV-ITEC Prochem Gel Dock System (Whitehead Scientific) was used to view the gel.

2.4.1.9 RNA transcript quantification and preparation

To quantify the RNA transcript the Qubit® 2.0 fluorometer (Thermo Fisher Scientific, Waltham, USA) was used. The Qubit® RNA High Sensitivity (HS) Assay Kit (Thermo Fisher Scientific, Waltham, USA) was used for the quantification of the RNA transcript. This kit includes the concentrated assay reagent, dilution buffer, and prediluted RNA standards. The manufacturer's instructions were followed to determine the quantity of the RNA transcript. In summary: the concentrated assay reagent was diluted with the buffer provided, the RNA transcript was added to the diluted assay reagent (any volume from 1-20 µL, in this case 2 µL of the sample was added) and the concentration of the sample was then read using the Qubit® 2.0 fluorometer (Thermo Fisher Scientific).

To determine the exact RNA copy number from the concentration obtained from the Qubit® 2.0 fluorometer (Thermo Fisher Scientific), an online calculator called Endmemo (Endmemo, 2016) was used. After determining the RNA copy number, the RNA transcript was first diluted down to 1 x 10^{10} copies/µL and then from this, ten-fold serial dilutions were performed to reach a concentration of 1 x 10^{6} copies/µL (Figure 2.6). Serial dilutions were performed with TDR buffer which was prepared by combining 5 mM Tris-hydrogen chloride (HCl) (Sigma-Aldrich, St. Louis, USA), 100 mM dithiothreitol (DTT, Thermo Fisher Scientific, Waltham, USA) and 10 000 units/mL recombinant RNasin ribonuclease inhibitor (Promega Corp., Madison, USA) (see Addendum A7). From the 1 x 10^{6} copies/µL tube, 96 single-use aliquots of 25 µL each were made, and the aliquots were stored at -80°C until future use in the RNA RT-qPCR assay as the HIV-1 subtype C *integrase* RNA standard.

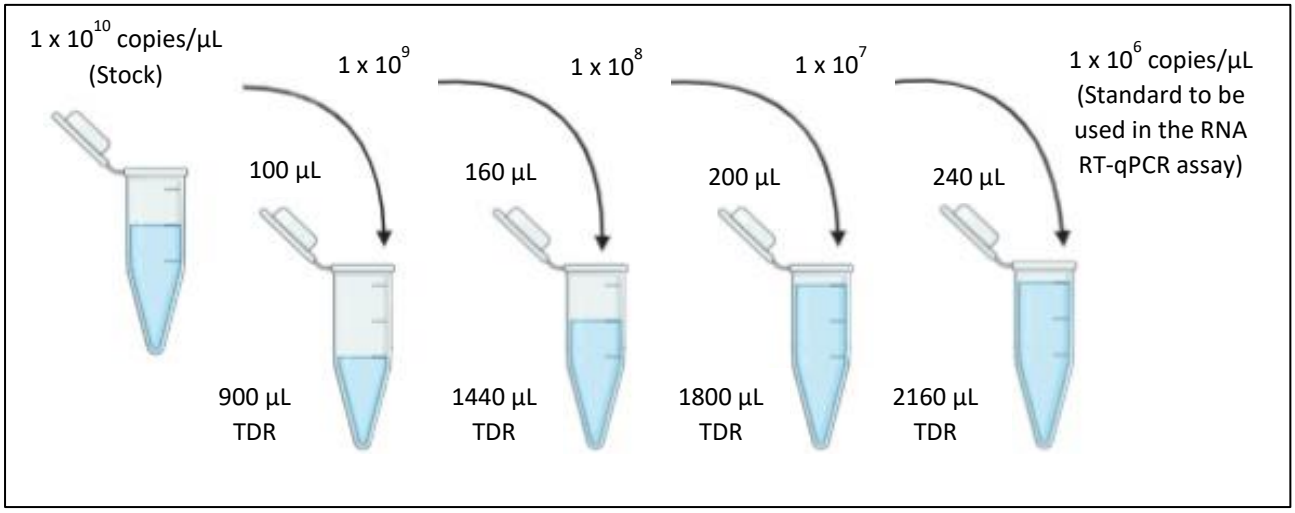

Figure 2.6: Ten-fold serial dilution series of the RNA standard to be used in the RNA RT-qPCR assay

For the serial dilutions, TDR buffer was used. (Picture was created with BioRender.com).

2.4.2 Testing the RNA standard curves for DNA signal to evaluate DNase I efficiency

The RNA standard was evaluated for DNA signal by performing a RNA RT-qPCR assay with the RNA standard and a no-reverse transcriptase (NRT) control of the RNA standard. The RNA RT-qPCR assay is a two-step qPCR method, meaning that complimentary DNA (cDNA) is created first from the RNA transcript in a separate reverse transcription reaction and then the qPCR reaction master mix will be added to the cDNA mix for the real-time reaction. This experiment was performed twice. For each experiment, a cDNA master mix was prepared for both the reverse transcriptase (RT) and NRT control reactions

(Addendum A8). A plate layout of these two experiments can be seen in Figure 2.7. The RNA standard which was prepared earlier in Section 2.4.1 was diluted down from 1 x 10^6 copies/µL to 30 copies/well (10 µL). To perform the serial dilutions of the RNA standard, a total of eight tubes were set out where 180 µL of TDR was dispensed into the first three tubes and 50 µL was dispensed into the last five tubes. Then 20 µL of the HIV-1 RNA *integrase* standard was transferred to the first tube labelled 1 x 10^6 copies/10 µL from the stock tube (1 x 10^7 copies/10 µL), the pipette tip was changed before mixing 10X by aspirating and dispensing using the micropipette. After that, 20 µL of this new dilution was transferred to the next tube labelled 1 x 10^5 copies/10 µL and this process was repeated until 1 x 10^4 copies/10 µL was reached. From this concentration, 25 µL of the new dilution was transferred to the fourth tube that contained 50 µL TDR to reach a concentration of 3000 copies/10 µL. This was repeated until 30 copies/10 µL was reached. The dilutions performed from 1 x 10^7 copies/10 µL down to 1 x 10^4 copies/10 µL were ten-fold serial dilutions; whereas the dilutions performed from 1 x 10^4 copies/10 µL down to 30 copies/10 µL were 1 to 3.2 fold dilutions and because 3.2 is approximately equal to $10^{1/2}$, every second dilution was therefore a ten-fold dilution. After the RNA *integrase* standard dilutions had been prepared for the RNA RT-qPCR assay, 10 µL of each dilution from 1 x 10^5 copies/10 µL to 30 copies/10 µL was dispensed in triplicate for both the RT and NRT reactions according to the plate layout in Figure 2.7. The plate was tightly sealed with a clear plastic film and centrifuged briefly to ensure that all the fluid was at the bottom of each well for increased accuracy. For cDNA synthesis, the plate was loaded onto the CFX Connect real-time PCR machine (Bio-Rad Laboratories, Hercules, USA). This was not a qPCR reaction, but a qPCR machine was used for more optimised workflow in the laboratory when it came to adding the real-time master mix after the cDNA step was completed. The cycle conditions for the cDNA synthesis step can be seen in Addendum A8.

	1	2	3	4	5	6	7	8	9	10	11	12
A	30 NRT			100 NRT			300 NRT			1000 NRT		
B	3000 NRT			10 000 NRT			100 000 NRT			HIV NTC NRT		
C												
D												
E												
F												
G	30 RT			100 RT			300 RT			1000 RT		
H	3000 RT			10 000 RT			100 000 RT			HIV NTC RT		

Figure 2.7: Plate layout for RNA RT-qPCR assay to evaluate DNase I efficiency

The RNA standard was diluted down to 30 copies/10 µL. From the dilutions, all of the diluted standard from 100 000 copies/10 µL to 30 copies/10 µL was included in the assay The RNA standard dilutions were assayed in triplicate for both the NRT control reactions (to evaluate DNA signal) and also for the RT reactions (DNA and RNA signal).

After the completion of the cDNA step, the qPCR master mix was prepared as observed in Addendum A8, the clear plastic film was gently pulled off from the 96-well qPCR plate (Bio-Rad Laboratories, Hercules, USA) and 20 µL of this master mix was added to all of the RT and NRT wells for real-time amplification of the cDNA. A new clear plastic film was used to cover the 96-well real-time PCR plate (Bio-Rad Laboratories). The plate was centrifuged briefly to remove any air bubbles that could interfere with the qPCR and the plate was loaded onto the CFX Connect real-time PCR machine (Bio-Rad Laboratories) following the cycle conditions as observed in Addendum A8. Once the run was completed, the results were analysed using Bio-Rad CFX Manager version 3.0 software (Bio-Rad Laboratories, Hercules, USA).

2.5 Implementation of the VOA

2.5.1 Preparation of the T cell growth factor

T cell growth factor is an essential reagent needed to perform the VOA. Growth factors are signalling molecules which stimulate the production and development of T cells. A number of the growth factors have been discovered, among them many members of the IL family, for example: IL-2 (Oppenheim, 2007), IL-7 (Chazen et al., 1989) and IL-15 (Steel et al., 2012). Buffy coats from different HIV-1-uninfected donors were obtained from the WCBS to prepare the TCGF. The protocol for the preparation of the TCGF was adapted from Laird, Rosenbloom, Lai, Siliciano & Siliciano, 2016.

First, PBMCs were isolated from the buffy coats by Ficoll density centrifugation. The buffy coats were diluted 1:1 with wash media (2% qualified heat-inactivated and gamma-irradiated Foetal Bovine Serum [HI & GI FBS] [Thermo Fisher Scientific, Waltham, USA] added to 1X Dulbecco's phosphate-buffered saline [DPBS] [Thermo Fisher Scientific, Waltham, USA] [Addendum B1]). This was followed by layering the diluted buffy coats on Histopaque®-1077 (Sigma-Aldrich, St. Louis, USA) for centrifugation. The Jouan BR4i centrifuge (Jouan, Saint-herblain, France) with a rotor radius of 161 mm was used to separate the PBMCs from the buffy coat suspension at 1623 RCF for 30 minutes with the brakes off. Using a 3 mL disposable Pasteur pipette (Lasec® Group, Cape Town, SA) the band of PBMCs were removed and added to wash media for a washing step at 721 RCF for 15 minutes with the brakes off. The washing step was repeated as the original volume of this sample was more than 15 mL (samples of 15 mL or less were only washed once). The supernatant was poured off and the pellet was resuspended in the appropriate volume of wash media to prepare it for cell counting. Trypan Blue (Bio-Rad Laboratories, Hercules, USA) was used to stain the cells for counting purposes and the TC20™ Automated cell counter (Bio-Rad Laboratories, Hercules, USA) was used. Trypan Blue (Bio-Rad Laboratories) is used to determine the number of viable cells present in a cell suspension. It is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as Trypan Blue, Eosin, or propidium, whereas dead cells do not (Strober, 2001). For cell counting purposes, 50 µL of Trypan Blue (Bio-Rad Laboratories) was mixed with 50 µL of the cells suspension and then 10 µL of this mixture was pipetted onto both sides of a TC20™ dual-chamber counter slide (Bio-Rad Laboratories, Hercules, USA). The outside of the slide was wiped with 70% general

use ethanol and then placed into the TC20™ Automated cell counter (Bio-Rad Laboratories). The lower gate was set to 6 µm and the upper gate was set at 17 µm. The cells were counted four times by reading it twice on each side of the slide and the average live cell count was used to determine how much TCGF cell culture media would be necessary to resuspend the cells at a final concentration of 5 x 106 cells/mL. The TCGF media was prepared by adding 2.5% HI Human Serum AB male HIV tested, product code: S4190, (Biowest, Nuaillé, France) and 1X Penicillin-Streptomycin (Biosciences, Dublin, Ireland) to Roswell Park Memorial Institute (RPMI) 1640 medium with L-Glutamine and phenol red (Lonza Group Ltd, Basel, Switzerland) (see Addendum B2). After counting the cells, the cells were centrifuged at 406 RCF for 10 minutes with the brakes off. The wash media was decanted and the cells were combined at 5 x 106 cells/mL in TCGF cell culture media. The cell suspension was added at a final concentration of 0.33 x 106 cells in a final volume of 75 mL TCGF cell culture media in a Nunc™ EasYFlask™ 175 cm2 cell culture flask (vented cap, tissue culture [TC]-treated) (Thermo Fisher Scientific, Waltham, USA). The cells were incubated overnight in a humidified CO2 Nuaire NU-5510E (Nuaire, Plymouth, USA) incubator at 37°C, 5% CO2 with the flasks lying flat.

The following day the cells were activated by adding Remel™ Purified PHA (Thermo Fisher Scientific, Waltham, USA) to a final concentration of 2 µg/mL and PMA (Sigma-Aldrich, St. Louis, USA) to a final concentration of 5 ng/mL. After the addition of PHA (Thermo Fisher Scientific) and PMA (Sigma-Aldrich), the cells were incubated in a humidified CO_2 Nuaire NU-5510E (Nuaire) incubator at 37°C, 5% CO_2 for 4 hours. The culture supernatant was aspirated off and the settled PBMCs were washed three times with 37°C pre-warmed wash media to remove any residual PHA (Thermo Fisher Scientific) and PMA (Sigma-Aldrich). Cells were washed gently so as to not remove any cells attached to the surface of the Nunc™ EasYFlask™ 175 cm^2 cell culture flask (vented cap, TC-treated) (Thermo Fisher Scientific). After the washing step, fresh 37°C pre-warmed TCGF cell culture media was added back to the Nunc™ EasYFlask™ 175 cm^2 cell culture flask (vented cap, TC-treated) (Thermo Fisher Scientific) and the cells were incubated in a humidified CO_2 Nuaire NU-5510E (Nuaire) incubator at 37°C, 5% CO_2 for 40 hours.

After 40 hours incubation, the cell culture supernatant from the activated, settled PBMCs was collected from the Nunc™ EasYFlask™ 175 cm^2 cell culture flasks (vented cap, TC-treated) (Thermo Fisher Scientific) and centrifuged at 1127 RCF for 10 minutes with no

brake or acceleration to remove any cell debris. The cell culture supernatant was then filter-sterilised using a 0.45 µm Nalgene filter unit (Thermo Fisher Scientific, Waltham, USA) to isolate only the growth factors from the cell culture supernatant. This filtered TCGF was stored in 10 mL aliquots at -20°C for use in the VOA.

2.5.1.1 Evaluating the efficiency of the T cell growth factor

To determine the optimal amount of TCGF that had to be added to the cell culture medium to produce maximal cell growth during the VOA, the efficiency of the TCGF was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) Cell Proliferation Assay Kit (Colorimetric) (Abcam®, Cambridge, UK). Colometric analysis is used to determine the concentration of substances that absorb light in the visible region (400-800 nm) by measuring the intensity of light, which depends on the colour. By adding the MTS reagent, cell proliferation can be measured based on the reduction of MTS tetrazolium compound by viable cells to generate a coloured formazan product that is soluble in cell culture media. This conversion is thought to be carried out by Nicotinamide Adenine Dinucleotide Phosphate Hydrogen (NADPH)-dependant dehydrogenase enzymes in metabolically active cells. The formazan produced by viable cells can be quantified by measuring the absorbance at optical density (OD) of 490-500 nm. Typically, no more than 2% of TCGF is required to stimulate maximal cell proliferation, at higher concentrations activated CD8+ T cells can introduce the production of more chemokines which can inhibit viral replication in the VOA (Laird et al., 2016).

To test the efficiency of the TCGF, 40 mL blood was collected from an HIV-1 negative patient. Total PBMCs were isolated by Ficoll density centrifugation followed by cell counting via Trypan Blue exclusion as described in Section 2.5.1. The average live cell count was used to determine how much cell culture media will be necessary to resuspend the cells at a final concentration of 0.50×10^6 cells/mL. This cell culture media for human PBMCs were prepared as follows: to RPMI 1640 medium with L-Glutamine and phenol red (Lonza Group Ltd), add 20% HI & GI FBS (Thermo Fisher Scientific) and 100 U/mL Gibco™ Recombinant human IL-2 (Thermo Fisher Scientific, Waltham, USA) (refer to Addendum B2). After counting the cells, they were centrifuged at 521 RCF for 10 minutes with no brake or acceleration using the Jouan BR4i centrifuge (Jouan) with a rotor radius of 161 mm. The supernatant was decanted, and the pellet was resuspended in human PBMCs cell culture

media to a final concentration of 0.50 x 10^6 cells/mL. Different concentrations of TCGF, starting from 1% to 5% were added in triplicate with 0.50 x 10^6 cells/mL PBMCs, in cell culture media, at a final volume of 100 µL per well in a 96-well TC-treated microplate (Corning® Incorporated, New York, USA) as shown in Figure 2.8. The cells were incubated for three days at 37°C, 5% CO_2 in a Nuaire NU-5510E (Nuaire) incubator.

After the three days incubation, 20 µL/well of the MTS reagent (MTS Cell Proliferation Assay Kit [Colorimetric] [Abcam®]) was added to each well. The cells were incubated for 2 hours at 37°C, 5% CO_2 in the Nuaire NU-5510E (Nuaire) incubator. The absorbance was measured using the EZ Read 400 microplate reader (Biochrom Ltd., Cambridge, UK) at OD 492 nm and the data was acquired using Galapagos software version 1.1.0.0 (Biochrom Ltd., Cambridge, UK). The concentration of TCGF that gave the highest OD reading at 492 nm was the most efficient and was the concentration that was selected for use in the VOA.

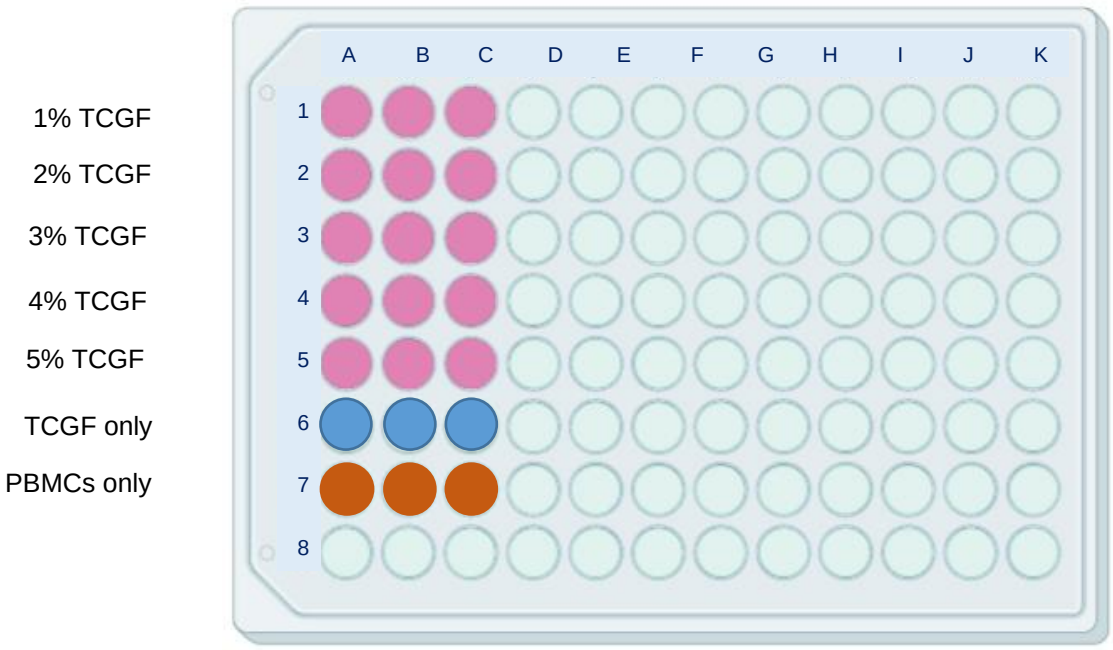

Figure 2.8: Different concentrations of TCGF added in triplicate on a TC-treated 96 well microplate (Corning® Incorporated) with PBMCs to evaluate TCGF efficiency

Different concentrations of the TCGF were added in triplicate with human PBMCs to evaluate the efficiency of the TCGF (this figure was created in BioRender.com).

2.5.2 Growing up MOLT-4 CCR5+ cells as target cells for the VOA

To propagate HIV-1 in the VOA, the MOLT-4 CCR5+ cell line was used. The MOLT-4 CCR5+ T cell line was obtained in the form of a single aliquot of 1 mL through the

NIH AIDS Reagent Program, NIAID, from Dr. Masanori Baba, Dr. Hiroshi Miyake, Dr. Yuji Iizawa (Baba et al., 2000). Due to only having one aliquot of the MOLT-4 CCR5+ T cell line, the cells had to be grown in culture to cryopreserve multiple 1 mL aliquots at a concentration of 5 x 10^6 cells/mL for future use in the VOA.

To accomplish this, the 1 mL aliquot was thawed quickly (approximately two minutes) in a 37°C bead bath. The thawed cells were added dropwise to 37°C prewarmed Gibco™ DPBS without Calcium and Magnesium (Thermo Fisher Scientific) to wash off the cryopreservation media (10% dimethyl sulfoxide [DMSO, Sigma-Aldrich, St. Louis, USA] and 90% HI & GI FBS [Thermo Fisher Scientific]) (Addendum B1) at 125 RCF for seven minutes with the brake and acceleration off, using the Jouan BR4i centrifuge (Jouan) with a rotor radius of 161 mm. The supernatant was discarded, and the cell pellet was resuspended in 5 mL 37°C DPBS (Thermo Fisher Scientific) for cell counting. The Trypan Blue exclusion method, as described previously under Section 2.5.1, was used to count the cells. In this case the lower gate was set to 6 µm and the upper gate was set at 20 µm according to the cell size of the MOLT-4 CCR5+ cells. The average live cell count of the MOLT-4 CCR5+ cells were used to seed the cells at a concentration of 1.2-1.8 x 10^5 cells/mL in MOLT-4 CCR5+ cell culture recovery media (to RPMI 1640 medium without L-Glutamine and phenol red [Lonza Group Ltd, Basel, Switzerland] add 20% HI & GI FBS [Thermo Fisher Scientific] and 1X GlutaMAX™ [Biosciences, Dublin, Ireland] [Addendum B3]) in a Nunc™ EasYFlask™ 25/75 cm^2 cell culture flask (vented cap, TC-treated) (Thermo Fisher Scientific). The cells were incubated in a humidified CO_2 Nuaire NU-5510E (Nuaire) incubator at 37°C, 5% CO_2.

The cells were observed with an inverted ZEISS Primovert light microscope (Carl Zeiss AG, Oberkochen, Germany) every three to four days to ensure that there was no sign of visible contamination with bacteria (which would appear as like tiny granules moving between the cells), yeasts (yeasts would appear as individual ovoid or spherical particles that may bud off smaller particles) or molds (molds grow as multicellular filaments known as hyphae. Under a microscope, they would appear as thin, wisp-like filaments, and sometimes as denser clumps of spores). Contamination with any of these microorganisms may also make cell culture media appear turbid (Invitrogen, 2017). Occasionally, when the cells were observed with the ZEISS Primovert light microscope (Carl Zeiss AG), photographs would be taken with the Axiocam ERc 5s camera (Carl Zeiss AG, Oberkochen, Germany) for quality control purposes to enable possible troubleshooting should any morphological abnormalities

be observed. The photographs were analysed with ZEN Blue Edition Version 3.2.0 (Carl Zeiss AG, Oberkochen, Germany).

The MOLT-4 CCR5+ cells were sub-cultured, by diluting the cells 1:10, every three to four days (twice a week) only if the total live cell count (based on Trypan Blue exclusion) was between 1-2 x 10^6 cells/mL (if the cells were not ready to be passaged based on the total live cell count, the cell culture media would be replenished). It is also important to note that the MOLT-4 CCR5+ cell culture recovery media was only used to seed the cells, since it was noted that the cells grew better from cryopreservation without Penicillin-Streptomycin (Biosciences). However, due to a risk of contamination when the cells were ready for passage number one or when the cell culture media was replenished for the first time, MOLT-4 CCR5+ media was used. The MOLT-4 CCR5+ media contained the same supplements as the recovery media, except in this case Penicillin-Streptomycin (Biosciences) would also be added at a concentration of 1X (see Addendum B3).

If the cells were not ready to be passaged, the following was done to replenish the media: after counting the cells via Trypan Blue exclusion as mentioned before and finding that the total average live cell count was less than a 1 x 10^6 cells/mL, the cells in suspension would be centrifuged at 125 RCF for seven minutes with the brakes off. The cell culture supernatant was discarded, and the cell pellet was resuspended in the appropriate volume of 37°C pre-warmed MOLT-4 CCR5+ media, according to the size of Nunc™ EasYFlask™ cell culture flask (vented cap, TC-treated) (Thermo Fisher Scientific) used. Corning® Incorporated recommends using 0.2 to 0.3 mL medium for each square centimeter of culture vessel growth surface area. The cells in media were then added slowly to the appropriate size cell culture flask and placed in a humidified CO_2 Nuaire NU-5510E (Nuaire) incubator at 37°C, 5% CO_2. The cells were then checked again after three or four days.

Once the cells had reached a concentration of 1-2 x 10^6 cells/mL, they were passaged by performing the following steps: the cells in media was centrifuged at 125 RCF for seven minutes with the brakes off. The cell culture supernatant was discarded, and the cell pellet was resuspended in the same amount of 37°C pre-warmed MOLT-4 CCR5+ media as there originally was in the cell culture flask. The cells were then passaged into new cell culture flasks by diluting the cells 1:10 with fresh MOLT-4 CCR5+ media that was pre-warmed at 37°C. The cells were placed back into the humidified CO_2 Nuaire NU-5510E (Nuaire)

incubator at 37°C, 5% CO_2 until the media had to be replenished again or until the cells were ready for the next passage.

Multiple 1 mL aliquots at 5 x 10^6 cells/mL of the MOLT-4 CCR5+ cells were cryopreserved for future use in the VOA at a low passage number (passage number of five or below, these cells were cryopreserved at passage number five). These cells were cryopreserved at a low passage number as there is literature that showed that the passage number could affect the cell line's characteristics over time. Cell lines at high passage numbers experience changes in transfection efficiency, morphology, growth rates, protein expression and responses to stimuli. Therefore, it is better to use cells at lower passage numbers and to avoid too high passage numbers to ensure good quality data (Chang-Liu & Woloschak, 1997) (Wenger et al., 2004) (ATCC, 2007).

2.5.3 The VOA protocol used in this study

The protocol for the VOA was adapted from the Laird et al. (2016) protocol. An overview of the VOA can be seen in Figure 2.9, followed by a detailed description of each day in the VOA as depicted in the figure. The VOA is typically a 21-day assay, but it can be lengthened for up to 28 or 35 days, to isolate replication-competent virus from children.

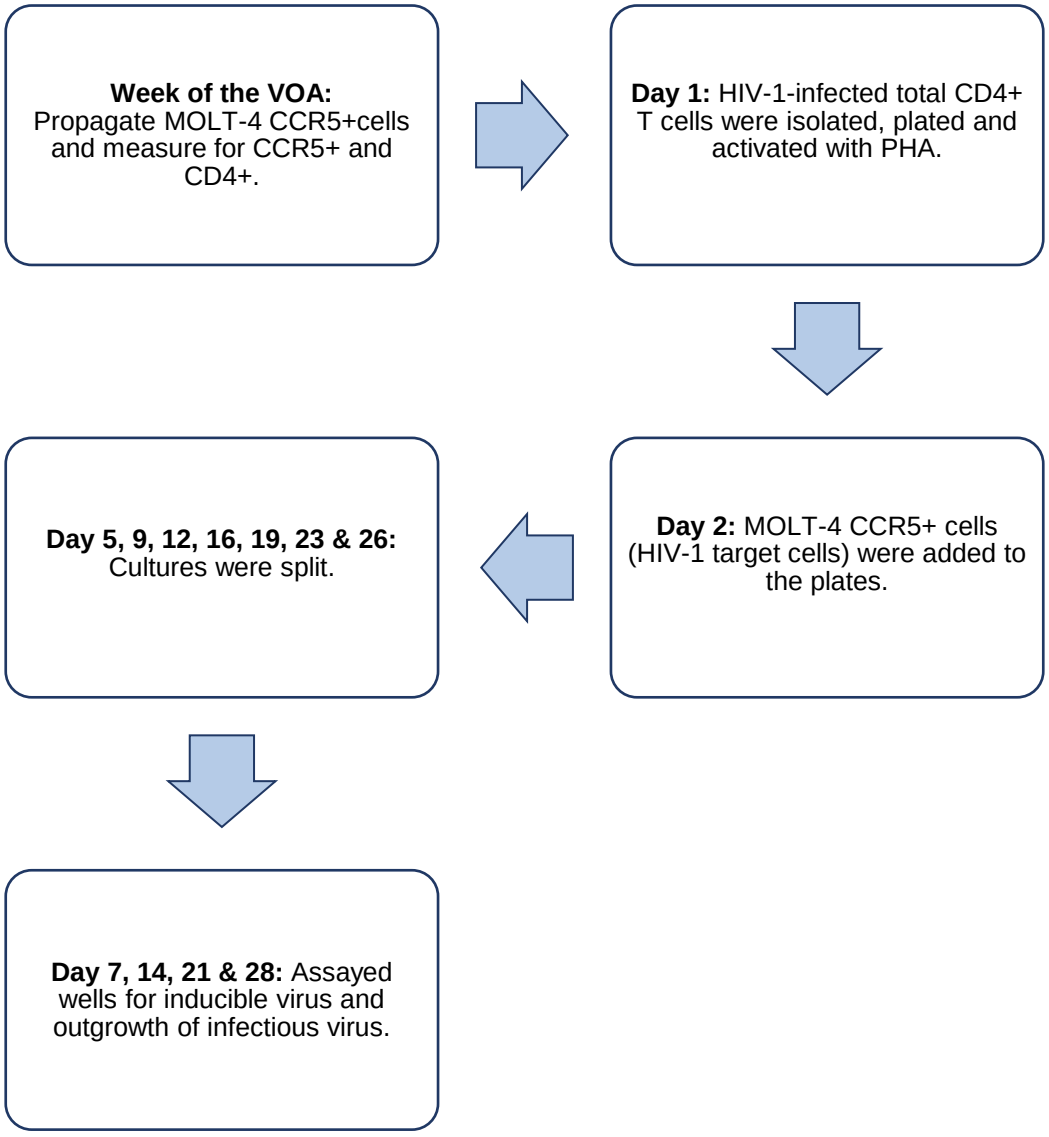

Figure 2.9: Overview of the 28 day VOA protocol

Week of the VOA:

Before starting the VOA, it is important to ensure that there are sufficient MOLT-4 CCR5+ cells in culture that will be used as the target cells for the expansion of HIV-1. Generally,

MOLT-4 CCR5+ cells were seeded and maintained in culture about two to three weeks before starting with the VOA (as explained under Section 2.5.2).

At the beginning of this project, it did not seem necessary to check the MOLT-4 CCR5+ cells for expression of the CD4+ receptor and for the CCR5+ co-receptor as Baba et al. (2000) have shown that these cells express both the CXCR4+ and CCR5+ co-receptors as well as the CD4+ receptor. It was only realised later (during troubleshooting the VOA) that testing the cells for expression of these receptors was necessary for quality control purposes.

Day one of the VOA:

Day one of the VOA involved preparation of the feeder cells, isolation of total CD4+ T cells from HIV-1-infected PBMCs, plating the cells together with the feeder cells and activating the cultures with PHA (Thermo Fischer Scientific). This was done as follows: for preparation of the feeder cells, 80-150 mL of ethylenediaminetetraacetic acid (EDTA) whole blood was obtained from healthy donors. The blood was irradiated by gamma-irradiation at 5000R in a Precision MultiRad160 X-irradiator (Precision X-Ray Inc., Branford, USA) for 14 minutes. Total PBMCs were isolated by Ficoll density centrifugation and counted via Trypan Blue exclusion as described before under Section 2.5.1. The PBMCs were then resuspended in super T cell medium (STCM) at a final concentration of 2.5 x 10^6 cells/mL. Super T cell medium (STCM) consisted out of 20% HI & GI FBS (Thermo Fisher Scientific), 1X GlutaMAX™ (Biosciences), 1X Penicillin-Streptomycin (Biosciences), 100 U/mL Gibco™ Recombinant IL-2 (Thermo Fisher Scientific) and 2% TCGF (In-house) added to RPMI 1640 medium without L-Glutamine and phenol red (Lonza Group Ltd) (refer to Addendum B3). To stimulate the cells to mitotically divide, PHA (Thermo Fisher Scientific) was added at a final concentration of 1 µg/mL. The cells were placed on ice until addition to the 6-well CytoOne® plate (TC-treated) (STARLAB, Hamburg, Germany) to prevent cell adhesion to the tubes.

The following step involved isolating total CD4+ T cells from the HIV-1-infected patients. Ficoll density gradient centrifugation was used to isolate PBMCs from EDTA whole blood. The total number of viable PBMCs had to be determined by Trypan Blue exclusion as described earlier under Section 2.5.1. The average live cell count of the PBMCs isolated were then used to determine the starting volume of the sample in EasySep™ Buffer (STEMCELL™ Technologies Inc., Vancouver, Canada) to obtain a starting concentration of 5 x 10^7 cells/mL within a volume range of 0.5-2 mL for total CD4+ T cell isolation. Total CD4+

T cells were isolated by immunomagnetic negative selection using the EasySep™ Human CD4+ T Cell Isolation Kit (STEMCELL™ Technologies Inc., Vancouver, Canada) with the EasyEights™ Magnet (STEMCELL™ Technologies Inc., Vancouver, Canada) following the 5 mL protocol according to the manufacturer's instructions. This kit targets non-CD4+ T cells for removal with antibodies recognising specific cell surface markers. Unwanted cells were labelled with antibodies and magnetic particles and separated without columns using the EasyEights™ Magnet (STEMCELL™ Technologies Inc.). Desired cells were aliquoted into a new tube and cells were then ready for downstream experiments (flow cytometry, culture or DNA/RNA extraction). An overview of the protocol can be seen in Figure 2.10.

After total CD4+ T cells were isolated, the HIV-1-infected total CD4+ T cells from each patient, together with the PHA stimulated feeder cells and 37°C pre-warmed STCM were plated in a 6-well CytoOne® plate (TC-treated) (STARLAB) as follows: 1 mL HIV-1-infected CD4+ T cells were plated at 1×10^6 cells/mL together with 4 mL feeder cells at 2.5×10^6 cells/mL (1×10^7 cells/4 mL) and 3 mL 37°C pre-warmed STCM, therefore, each well had a final volume of 8 mL. The plates were placed in a humidified CO_2 Heraeus Hera Cell incubator (Heraeus, Hanau, Germany) at 37°C, 5% CO_2 until day two of the VOA.

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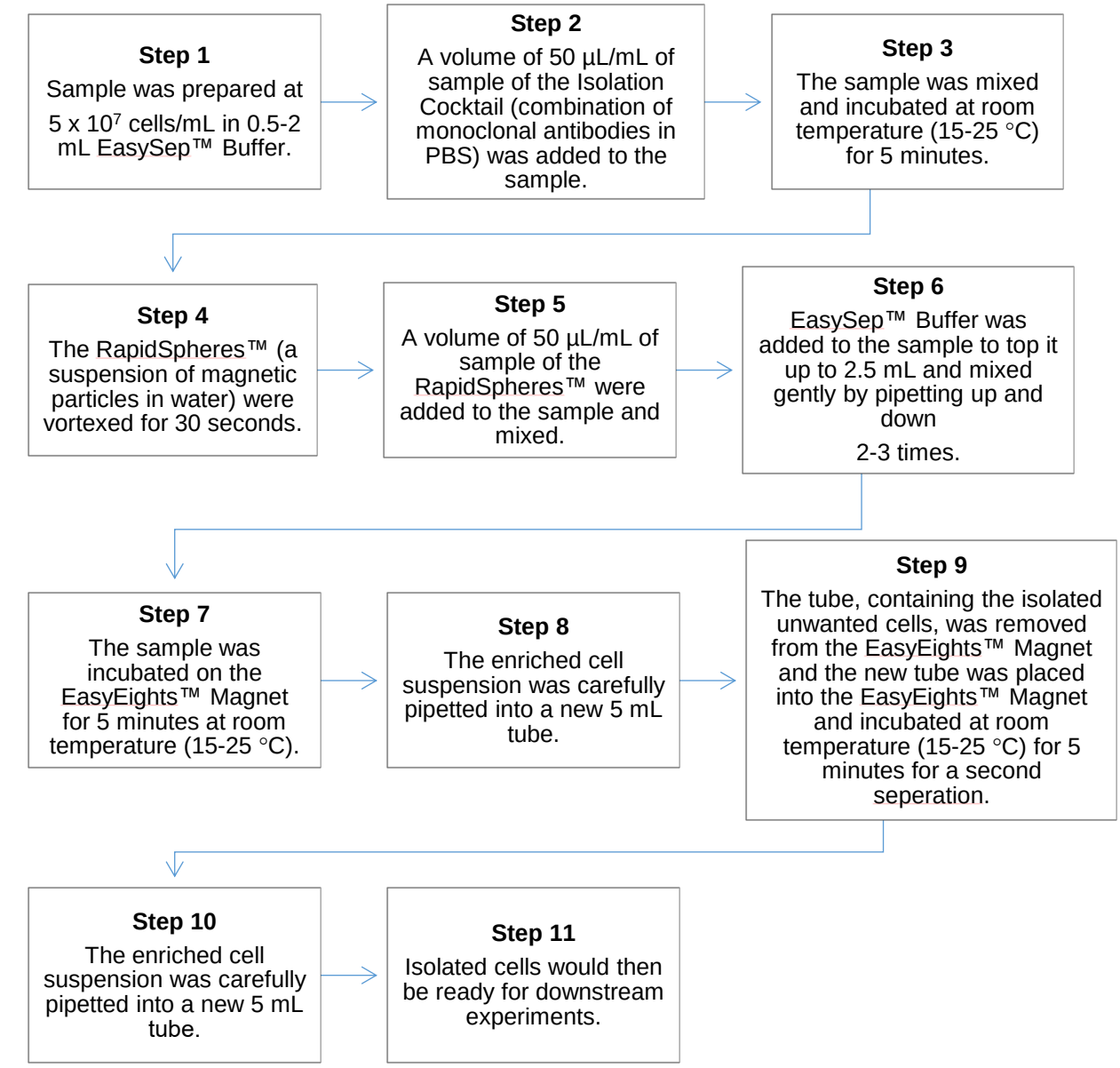


Figure 2.10: Overview of the CD4+ T cell isolation protocol from the EasySep™ Human CD4+ T Cell Isolation Kit (STEMCELL™ Technologies Inc.) with the EasyEights™ Magnet (STEMCELL™ Technologies Inc.)

Day two of the VOA:

Day two involved the removal of PHA (Thermo Fisher Scientific) from the cultures and the addition of the MOLT-4 CCR5+ cells as target cells for HIV-1 expansion. To minimise the toxicity of the PHA (Thermo Fisher Scientific), 6 mL of the cell culture media was removed, as little cells as possible was removed, and discarded. This was replaced with fresh, 37°C pre-warmed STCM and placed back into the humidified CO_2 Heraeus Hera Cell incubator

(Heraeus) at 37°C, 5% CO_2 for at least three hours to allow the cells to settle. Another 6 mL cell culture media was removed and discarded. This was replaced with an equal volume of MOLT-4 CCR5+ cells at 6.67 x 10^5 cells/mL (4 x 10^6 cells in 6 mL) in fresh, 37°C pre-warmed STCM. The plates were then placed back into the incubator at 37°C, 5% CO_2.

Day five, 12, 19 and day 26 of the VOA:

On day five of the assay, the cultures were split partially (removing less than half of the cells) to ensure the addition of fresh 37°C pre-warmed STCM to maintain the replication of HIV-1 by ensuring the MOLT-4 CCR5+ cells stayed viable. A total of 3 mL cell culture supernatant was removed from each well from each 6-well CytoOne® plate (TC-treated) (STARLAB) and discarded. The cells were then resuspended, and another 1 mL of the resuspended cells was removed and discarded. This was replaced with an equal volume of fresh 37°C pre-warmed STCM and incubated in the humidified CO_2 Heraeus Hera Cell incubator (Heraeus) at 37°C, 5% CO_2. This same procedure of splitting was repeated on days 12, 19 and 26 (and on day 33 if the VOA was 35 days long).

Day seven, 14, 21 and 28 of the VOA:

On day seven, 14, 21 and 28 (and day 35 for a 35-day VOA), a total volume of 2 mL supernatant was stored away at -80°C until measuring for inducible virus or outgrowth of virus with the in-house RNA RT-qPCR assay and theHIV-1 p24 antigen ELISA kit (PerkinElmer®, Waltham, USA). Before the supernatant was stored away, it was centrifuged at 2700 RCF in a Prism™ microcentrifuge (Labnet International Inc.) for 15 minutes to remove any residual cell debris. The supernatant was then aliquoted into working volumes of 1 mL, and four 250 µL aliquots.

To measure inducible virus or increased levels of cell-free RNA from the cell culture supernatant, RNA had to be extracted first, followed by performing the in-house RNA RT-qPCR assay on the extracted HIV-1 RNA. For performing the RNA RT-qPCR assay, the same protocol was followed as described earlier under Section 2.4.2. The NRT and RT master mix for the cDNA reaction would differ for each RNA RT-qPCR assay, depending on the number of reactions, and the same would be applicable for the real-time master mix. The plate layout would also look different, see Figure 2.11 compared to the plate layout in Figure 2.7.

To measure for infectious virus the HIV-1 p24 ELISA kit (PerkinElmer®) was used, following manufacturer's instructions. This kit measures the HIV-1 p24 core antigen in 200 µL cell culture supernatant. Samples were transferred to microplate wells coated with a mouse monoclonal antibody to HIV-1 p24. If there was any HIV-1 p24 in the sample, the antibody would capture it. The captured antigen would then complex with biotinylated polyclonal antibody to HIV-1 p24, followed by a streptavidin-horseradish peroxidase (HRP) conjugate. The resulting complex would then be detected by incubation with ortho-phenylenediamine-HCl (OPD) which would produce a yellow colour that would be directly proportional to the amount of HIV-1 p24 captured.

	1	2	3	4	5	6	7	8	9	10	11	12
A B	1 NRT	2 NRT	3 NRT	4 NRT	5 NRT	6 NRT	7 NRT	8 NRT	9 NRT	10 NRT	11 NRT	12 NRT
C D E	1 RT	2 RT	3 RT	4 RT	5 RT	6 RT	7 RT	8 RT	9 RT	10 RT	11 RT	12 RT
F												
G	30			100			300			1000		
H	3000			10 000			100 000			HIV NTC		

Figure 2.11: General plate layout for the RNA RT-qPCR assay when measuring for inducible virus and exponential outgrowth of virus from VOA cell culture supernatant

Each patient sample was tested in duplicate for the NRT control reaction to evaluate DNA signal from the patient sample. Each patient sample was also assayed in triplicate for the RT reaction to measure for inducible virus and exponential viral outgrowth. The RNA standard dilutions were also run in triplicate for each dilution from 100 000 copies/10 µL to 30 copies/10 µL to generate a standard curve for analysis.

Day nine, 16 and 23 of the VOA:

Day nine, 16, 23 (for a 28 day VOA) and 30 (for a 35 day VOA) involved a full culture split. A full split was done by resuspending the cells and removing and discarding half of the total volume of cell culture media (4 mL) and replacing it with fresh 37°C pre-warmed STCM. After splitting the cells, the cells were placed back into the humidified CO_2 incubator (Heraeus) at 37°C, 5% CO_2.

2.5.4 Attempt at validating the VOA by using fresh EDTA blood from two viraemic patients

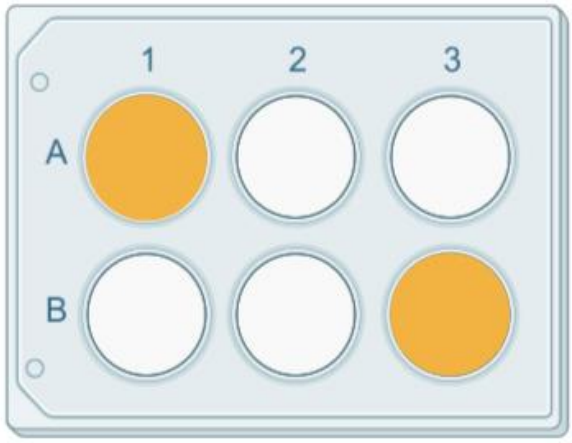
Initially, it was thought best to validate the VOA by using two viraemic HIV-1-infected patients. Information on each patient is presented in Table 2.2. The VOA was performed for 35 days on these patients, following the steps as described under Section 2.5.3. Figure 2.12 shows how the total CD4+ T cells isolated from the two viraemic patient samples were plated on the 6-well CytoOne® plates (TC-treated) (STARLAB) together with the PHA stimulated feeder cells and 37°C pre-warmed STCM. There were some differences in the VOA protocol steps in this experiment: on day one of the VOA, the feeder cells were only irradiated for 5 minutes and the cells were also not split on any of the splitting days (day five, nine, 12, 16, 19, 23, 26, 30 and day 33) during the 35 day assay. The cells in the patient wells as described in Figure 2.12 were counted via Trypan Blue exclusion (see Section 2.5.1, in this case the gates were set at 6 µm and 20 µm to include patient CD4+ T cells, feeder cells and MOLT-4 CCR5+ cells) to determine the viability percentage of the total cell population in these wells on days 14, 21 and 35. On days seven, 14, 21, 28 and 35, cell culture supernatant was first measured for p24 to assay for infectious virus with the HIV-1 p24 ELISA kit (PerkinElmer®) before measuring for increased levels of cell-free RNA. To measure for exponential outgrowth of virus from cell-free RNA in these two viraemic patients, the viral load in copies/mL was measured from 1 mL cell culture supernatant using a routine diagnostic test: COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test, v2.0 (Roche Holding AG, Basel, Switzerland). This test was chosen to measure for exponential increase in RNA instead of the in-house implemented RNA RT-qPCR assay, because this was an automated test and a fast answer was needed. Two conserved regions in the HIV-1 genome are targeted by this test: *gag* and LTR. The COBAS® AmpliPrep Instrument (Roche Holding AG, Basel, Switzerland) was used for automated sample processing and the COBAS® TaqMan® Analyzer (Roche Holding AG, Basel, Switzerland) for automated sample amplification and detection. The test could quantitate HIV-1 RNA over a range of 20-10 000 000 copies/mL. Amplilink Software Version 3.4.0 (Roche Holding AG, Basel, Switzerland) was used for analysis.

This validation experiment with the two viraemic patients did not produce the expected results, as no infectious virus was recovered. Therefore it was decided to troubleshoot the validation process by optimising the recovery of total CD4+ T cells from patient PBMCs, as well as to determine whether the MOLT-4 CCR5+ cells were permissive to HIV-1 infection.

Table 2.2: Characteristics of each patient used for the first validation attempt of the VOA

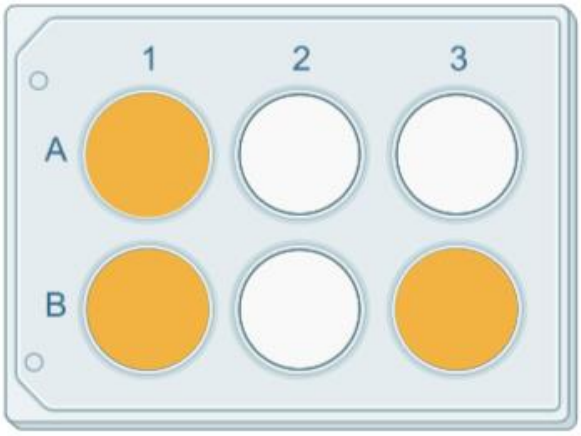
Characteristic	**Patient 1**	**Patient 2**
Age	Adult (>18 years, exact age not known)	Post-CHER Child (13 years old)
Volume of EDTA whole blood obtained	40 mL	30 mL
Viral Load	10 000 copies/mL	43 000 copies/mL
Total Viable PBMCs	68.9 x 10^6 cells/mL	17 x 10^6 cells/mL
Total Viable CD4+ T cells[a]	0.06 x 10^6 cells/mL 0.09% of total viable PBMCs	1.54 x 10^6 cells/mL 9% of total viable PBMCs
CD4+ T cell count BD Multitest™ kit[b]	3 CD4+ T cells/µL	582 CD4+ T cells/µL

[a]Total viable CD4+ T cells were isolated by immunomagnetic negative selection from PBMCs using the EasySep™ Human CD4+ T Cell Isolation kit (STEMCELL™ Technologies Inc.). [b]Total CD4+ T cell counts were also measured straight from whole blood using the CD4+ T cell count BD Multitest™ kit (BD Biosciences, San Jose, USA). A normal CD4+ T cell count with the BD Multitest™ kit (BD Biosciences) was between 550 and 1250 cells/µL.



6-well CytoOne® plate (TC-treated) (STARLAB) for patient 1:

- Well A1: Patient 1 (contained 0.06 x 10^6 CD4+ T cells/mL of the patient as well as PHA stimulated feeder cells and 37 °C pre-warmed STCM (Addendum B3)).
- Well B3: NTC (Everything the same, except it contained no HIV-1-infected CD4+ T cells).



6-well CytoOne® plate (TC-treated) (STARLAB) for patient 2:

- Well A1: Patient 2, well 1 (contained 1 x 10^6 CD4+ T cells/mL together with the PHA stimulated feeder cells and 37 °C pre-warmed STCM (Addendum B3)).
- Well B1: Patient 2, well 2 (contained 0.54 x 10^6 CD4+ T cells and also PHA stimulated feeder cells and 37 °C pre-warmed STCM (Addendum B3)).
- Well B3: NTC (Everything the same, except it contained no HIV-1-infected CD4+ T cells).

Figure 2.12: Plate layout for two viraemic patients in the first attempt of validating the VOA

(Picture was created with BioRender.com).

2.5.5 Troubleshooting the validation of the VOA

2.5.5.1 Optimisation of total CD4+ T cell recovery from PBMC

Due to the limited available sample volume when working with children, it was decided to optimise the total CD4+ T cells recovered from PBMCs using the 5 mL protocol from the EasySep™ Human CD4+ T Cell Isolation Kit (STEMCELL™ Technologies Inc.) with the EasyEights™ Magnet (STEMCELL™ Technologies Inc.). This was done to improve the yield of total CD4+ T cells isolated, in order to plate more replicates of wells in the VOA. An overview of the kit's protocol can be seen in Figure 2.10 with a total of 11 steps.

A healthy donor (female, 25 years of age) donated 34 mL EDTA whole blood for each of the optimisation experiments. Before performing the optimisation experiments, PBMCs had to be isolated by Ficoll density centrifugation and the average live cell counts of the total PBMCs were obtained by Trypan Blue exclusion as described before under Section 2.5.1. Three optimisation experiments were performed.

In experiment one, step five of the protocol was modified. Double the amount of the Dextran RapidSpheres™ was added to the sample. Therefore, while only 50 µL/mL of the sample of the Human CD4+ T Cell Isolation Cocktail was added, 100 µL/mL of the Dextran RapidSpheres™ was added.

For experiment two, step nine of the protocol was modified by repeating the separation for a third time.

In the final optimisation experiment, step nine of the protocol was modified once again. After pipetting the isolated cells into a new tube, the tube that contained the unwanted cells were separated by adding 2.5 mL of the EasySep™ Buffer (STEMCELL™ Technologies Inc.) and mixing the unwanted cells that were bound to the antibodies and the magnetic particles gently in order to ensure that no CD4+ T cells were captured by the particles or antibodies. The enriched cell suspension from both tubes were pooled together for use in the VOA.

To determine which of the three optimisation experiments worked best, the percentage of total CD4+ T cells recovered from PBMCs in each experiment was determined and the experiment that gave the higher percentage of CD4+ T cell recovery was chosen.

2.5.5.2 Testing the MOLT-4 CCR5+ cells for expression of the CD4+ receptor and for the CCR5+ co-receptor

Since HIV-1 requires both the CD4+ receptor (Maddon et al., 1986) (Mcdougal et al., 1986) and either one of the CCR5+/CXCR4+ co-receptors for infection (Moore et al., 1997), it was decided to test the MOLT-4 CCR5+ cells for the expression of the CD4+ receptor and for the CCR5+ co-receptor (utilised by R5 HIV-1). For this study's purposes, the cells were not tested for the CXCR4+ co-receptor (utilised by X4 HIV-1), since HIV-1 subtype C is most prevalent in South Africa and the percentage of X4 viruses appear to be lower than R5

viruses, even in the late-stage of the disease (Jacobs et al., 2009) (Bekker et al., 2018) (Cilliers et al., 2003) (Esbjörnsson et al., 2010).

Stained samples of MOLT-4 CCR5+ cells were acquired on a BD FACSCanto™ II instrument (BD Biosciences, San Jose, USA) using BD FACSDiva™ Software (BD Biosciences, San Jose, USA). Phycoerythrin (PE) anti-human CD195 (CCR5+) (Clone HEK/1/85a) monoclonal antibody (BioLegend®, San Diego, USA) was used to stain for the CCR5+ co-receptor. The CCR5+ monoclonal antibody had an excitation maximum wavelength at 565 nm and it had a maximum emission wavelength of 578 nm. It was excited by the blue laser (488 nm) and detected with a 585/42 nm filter. For staining for the CD4+ receptor, the Brilliant™ Blue 515 (BB515) Mouse Anti-Human CD4+ (Clone RPA-T4) monoclonal antibody (BD Biosciences, San Jose, USA) was used. This monoclonal antibody had an excitation maximum near 490 nm and an emission maximum near 515 nm, it was excited by the blue laser (488 nm) and detected with a 530/30 nm filter. The recommended volume for both of these monoclonal antibodies were 5 µL per million cells in a 100 µL staining volume.

In order to determine the minimum volume for the PE CD195 (CCR5+) monoclonal antibody (Biolegend®) as well as for the BB515 Mouse Anti-Human CD4+ (Clone RPA-T4) monoclonal antibody (BD Biosciences) that would produce the greatest signal-to-background ratio, for MOLT-4 CCR5+ cells specifically, antibody titrations were performed. The MOLT-4 CCR5+ cells were resuspended at 20 x 10^6 cells/mL in staining buffer (2% HI & GI FBS [Thermo Fisher Scientific] and 1X DPBS [Thermo Fisher Scientific]) (Addendum B1). The titrations started with a 1:10 dilution and the monoclonal antibodies were serially diluted (2-fold) down to a 1:0.312 dilution. To determine the optimal volume of each monoclonal antibody, the median fluorescent intensity (MFI) value of both positive and negative fluorescent signals generated were required. Therefore, for each antibody there was a total of six titrations and three unstained controls were also included in the experiment. The antibody volume, for both CCR5+ and CD4+, that showed optimal separation between the positive and negative populations was used.

After determining the optimal volume of each antibody, voltration had to be performed to ensure that the photocurrent was captured correctly by the electronics system. In flow cytometry, the most commonly used detectors are the photomultiplier tubes (PMT). The

PMT reside in each channel of the flow cytometer and when the photons are converted into photocurrent, the PMT amplifies the signal which requires a steady-state application of voltage to the detector. Therefore, PMT sensitivity is controlled by the material used to construct it and the wavelength of light that enters, as well as the amount of voltage that is applied. Voltration is the optimisation of the applied voltage for each PMT detector to obtain the best quality data in each channel of the flow cytometer (https://www.thermofisher.com/za/en/home/references/newsletters-and-journals/bioprobes-journal-of-cell-biology-applications/bioprobes-78/photomultiplier-tube-pmt-optimization-attune-nxt-flow-cytometer.html). For each monoclonal antibody, eight different voltages were compared (250 V, 300 V, 350 V, 400, V 450 V, 500 V, 550 V and 600 V). To determine the optimal PMT voltage there are two formulas that can be used. In this case both were used as one is not preferred above the other. The first formula was the separation index (see Addendum B4) and the second formula was the stain index (see Addendum B4). These formulas were used to draw voltration curves for each antibody. The optimal PMT voltage for each antibody would be at the point where a plateau was reached.

From here on, every time the VOA was performed, the MOLT-4 CCR5+ cells would be tested for the expression of the CD4+ receptor and the CCR5+ co-receptor via flow cytometry using the optimal PMT voltage conditions as well as the optimal antibody volumes. This was done to ensure that the cells were expressing these receptors as cell lines' characteristics can change in culture over time as mentioned before (Chang-Liu & Woloschak, 1997) (Wenger et al., 2004) (ATCC, 2007). If the MOLT-4 CCR5+ cells only expressed 20% or less of the CD4+ receptor, and 50% or less of the CCR5+ co-receptor, a new vial of the MOLT-4 CCR5+ cells would be thawed, grown and stained again for the CCR5+ co-receptor and the CD4+ receptor.

2.5.5.3 Testing whether the MOLT-4 CCR5+ cells were permissive to HIV-1 infection

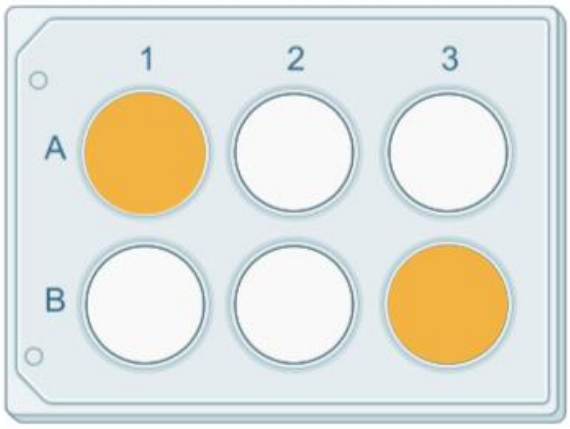
In attempt to further validate the VOA it was thought best to test whether the MOLT-4 CCR5+ cells were permissive to HIV-1 through infection of the cells with cell-free HIV-1 RNA from frozen plasma aliquots of two viraemic (viral load of >500 copies/mL) patient samples from the Post-CHER cohort. Details on these patients are presented in Table 2.3. For each of these two patient samples, three frozen plasma aliquots (at 1 mL each) were used for this experiment. The three 1 mL aliquots of each patient were pooled together to add to one

VOA well on a 6-well plate (STARLAB). Each sample also included an NTC. To be able to test the MOLT-4 CCR5+ cells for permissivity to HIV-1, the VOA, as explained under Section 2.5.3, was performed for 28 days with some modifications made to it. The modifications were as follows: day one of the VOA was excluded as cell-free plasma was used. Day two of the VOA became day one and on this day, the pooled frozen plasma aliquots from both samples were added to a well on a 6-well CytoOne® plate (TC-treated) (STARLAB) The layout of the plates for each patient sample can be seen in Figure 2.13. In this experiment the cells were also not split on days five, nine, 12, 16, 19, 23 or 26 during the 28 days. The viability of the MOLT-4 CCR5+ cells from the wells on the 6-well plates (STARLAB) that contained the frozen plasma from the patient samples as seen in Figure 2.13 were determined via Trypan Blue exclusion as described under Section 2.5.1 (with the lower gate set at 6 µm and the upper gate set at 20 µm) on days seven, 14, 21 and 28. To be able to determine whether the MOLT-4 CCR5+ cells were indeed infected with HIV-1 from the cell-free frozen plasma, outgrowth of HIV-1 was assayed by the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test, v2.0 (Roche Holding AG) on 1 mL cell culture supernatant from days seven, 14, 21 and 28. Cell culture supernatant were simultaneously assayed for infectious HIV-1 by measuring for the HIV-1 p24 antigen with the HIV-1 p24 ELISA kit (PerkinElmer®) per manufacturer's instructions from 200 µL cell culture supernatant on days seven, 14, 21 and 28. If there was an increase of the HIV-1 viral load over time or if there was positive p24 detection noticed by a colour change to yellow (as per the p24 ELISA kit's [PerkinElmer®] instructions) it would mean that the MOLT-4 CCR5+ cells were permissive to HIV-1 infection.

Table 2.3: Post-CHER patients selected to test the permissivity of the MOLT-4 CCR5+ cells

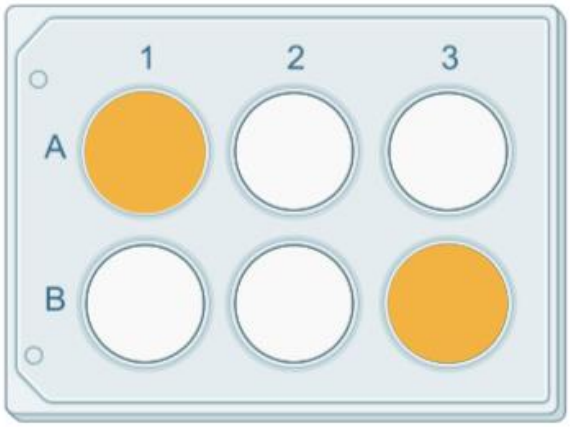
Patient ID	Date of birth	Gender	Date of sample collection	Visit number[a]	Viral Load (copies/mL)[b]
334846	16/06/2006	Male	18/02/2019	6	42 727
360582	11/11/2005	Male	25/05/2019	7	3218

[a]Patients of the post-CHER study had follow up visits where clinical assessments were done, such as monitoring CD4+ T cell counts and performing viral load tests. [b]The viral load for these patients were measured with the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test, v2.0 (Roche Holding AG) from 1 mL EDTA plasma.



6-well CytoOne® plate (TC-treated) (STARLAB) for patient 334846:

- Well A1: Pooled frozen plasma aliquots (from three aliquots) added to 4 x 106 MOLT-4 CCR5+ cells in 5 mL STCM (Addendum B3). The total volume was 8 mL.
- Well B3: NTC (Contained no HIV-1-infected plasma).



6-well CytoOne® plate (TC-treated) (STARLAB) for patient 360582:

- Well A1: Pooled frozen plasma aliquots (from three aliquots) added to 4 x 10^6 MOLT-4 CCR5+ cells in 5 mL STCM (Addendum B3). The total volume was 8 mL.
- Well B3: NTC (Contained no HIV-1-infected plasma).

Figure 2.13: Plate layout for the two viraemic Post-CHER patients to evaluate the permissivity of the MOLT-4 CCR5+ cells

(This picture was created in BioRender.com).

A second experiment was performed to test the permissivity of the MOLT-4 CCR5+ cells using fresh anonymised residual plasma samples obtained from 15 HIV-1 viraemic adults. Table 2.4 show information on these patients. All of these patients had a viral load of >10 000 copies/mL. Due to some of these patients not having exactly 1 mL of plasma and to increase chances of getting recoverable virus out, it was decided to pool the samples before infecting the MOLT-4 CCR5+ cells. An overview of the experiment set-up can be seen in Figure 2.14.

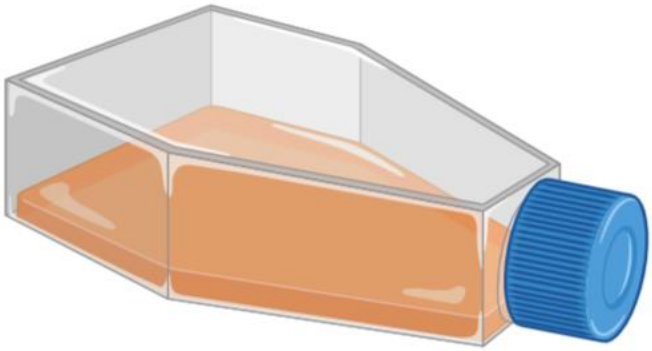
These cultures were incubated overnight in a Heraeus Hera Cell incubator (Heraeus) at 5% CO_2, 37°C to allow for infection and centrifuged the next day at 125 RCF for seven minutes with the brake and acceleration off, using the Jouan BR4i centrifuge (Jouan) with a rotor radius of 161 mm to remove the virus inoculum. To each of these cultures, the original volume of 37°C pre-warmed STCM was added again and the cultures were placed back into

the Heraeus Hera Cell incubator (Heraeus) at 5% CO2, 37°C. The viability percentage of the MOLT-4 CCR5+ cells (from the patient wells of the 6-well plate [STARLAB] as well as the two 75 cm2 cell culture flasks [Thermo Fisher Scientific] that contained fresh plasma from the viraemic adults) were determined on days three, seven and 10 via Trypan Blue exclusion as explained under Section 2.5.1 (the lower gate was set at 6 µm and the upper gate wat set at 20 µm). Supernatants in working volumes of 1 mL, and four 250 µL aliquots were removed on days three, seven and 10 and centrifuged at 2700 RCF in a Prism™ microcentrifuge (Labnet International Inc.) for 15 minutes to remove cell debris before storing it at -80°C until RNA extraction with the QIAamp® MinElute® Virus Spin Kit (Qiagen, Venlo, Netherlands) according to manufacturer's instructions. The in-house RNA RT-qPCR assay was used to measure the RNA extracted samples for exponential increase of HIV-1 RNA over time to determine if there was an increase of the virus and to confirm whether the MOLT-4 CCR5+ cells were permissive to HIV-1 or not. For the RNA RT-qPCR assay, the same protocol was followed as described earlier under Section 2.4.2, except for this experiment, samples were included in the assay and therefore the amount of cDNA and real-time master mixes had to be adjusted according to Addendum A8. Also, in this experiment, the RNA standards had no NRT controls since this was already evaluated before in Section 2.4.2. In this case, each sample was performed in duplicate to generate cDNA for the qPCR assay (the RT reaction) while there was one NRT control for each sample to evaluate for DNA signal. If there was a decrease in the cycle threshold (Ct) value over time according to the optimal threshold set for optimal efficiency (an efficiency of between 90% and a 110%, but closest to 100%) at the end of the real-time run (according to Life Technologies™), it would demonstrate that there was exponential increase in HIV-1 RNA which would prove permissiveness of the MOLT-4 CCR5+ cell line.

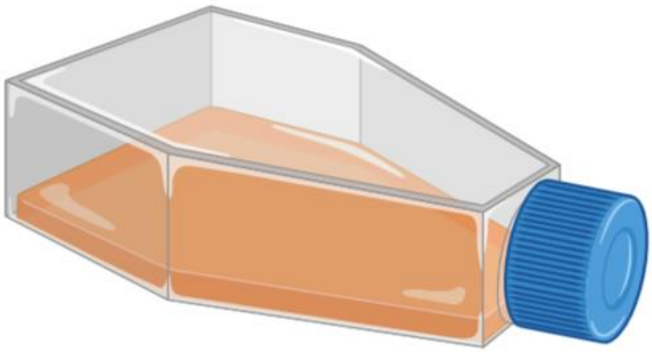
Table 2.4: Fifteen viraemic adults to test the permissivity of the MOLT-4 CCR5+ cells

Patient ID	Plasma volume obtained (mL)	Viral Load (copies/mL)*
001	0.70	52 388
002	0.60	97 411
003	1	26 134
004	1	530 299
005	1	64 282
006	1	17 526
007	0.70	12 468
008	0.40	31 599
009	0.50	161 757
010	1	10 224
011	1	51 494
012	1	10 000 000
013	1	32 434
014	0.50	30 380
015	1	213 371

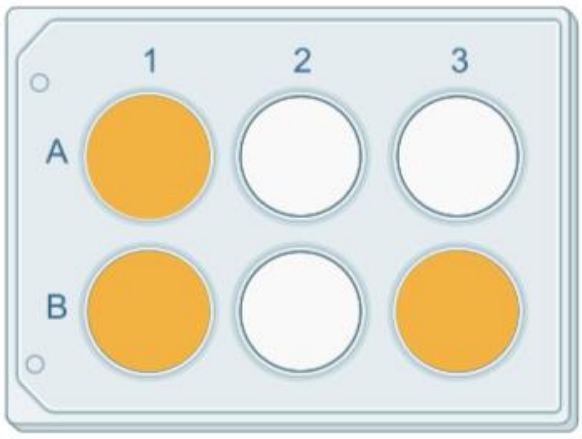
*The viral load for these patients were measured with the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test, v2.0. The test can quantitate HIV-1 RNA over the range of 48-10 000 000 copies/mL (Roche Holding AG).



Nunc™ EasYFlask™ 75 cm^2 cell culture flask A (vented cap, TC-treated) (Thermo Fisher Scientific):
Contained pooled plasma from patients 003, 004, 005, 006 and 011 (Table 2.4). This was added to 1 x 10^7 MOLT-4 CCR5+ cells in 15 mL STCM (Addendum B3).



Nunc™ EasYFlask™ 75 cm^2 cell culture flask B (vented cap, TC-treated) (Thermo Fisher Scientific):
Contained pooled plasma from patients 009, 010, 012, 013, 014 and 015 (Table 2.4). This was added to 1 x 10^7 MOLT-4 CCR5+ cells in 15 mL STCM (Addendum B3).



6-well CytoOne® plate (TC-treated) (STARLAB):

- Well A1: Pooled plasma from patients 001 and 002 (Table 2.4) added to 4 x 10^6 MOLT-4 CCR5+ cells in 6 ml STCM (Addendum B3).
- Well B1: Pooled plasma from patients 007 and 008 (Table 2.4) also added to 4 x 10^6 MOLT-4 CCR5+ cells in 6 ml STCM (Addendum B3).
- Well B3: NTC (Only contained 4 x 10^6 MOLT-4 CCR5+ cells in 6 mL STCM [Addendum B3] with an additional 2 mL STCM [Addendum B3]).

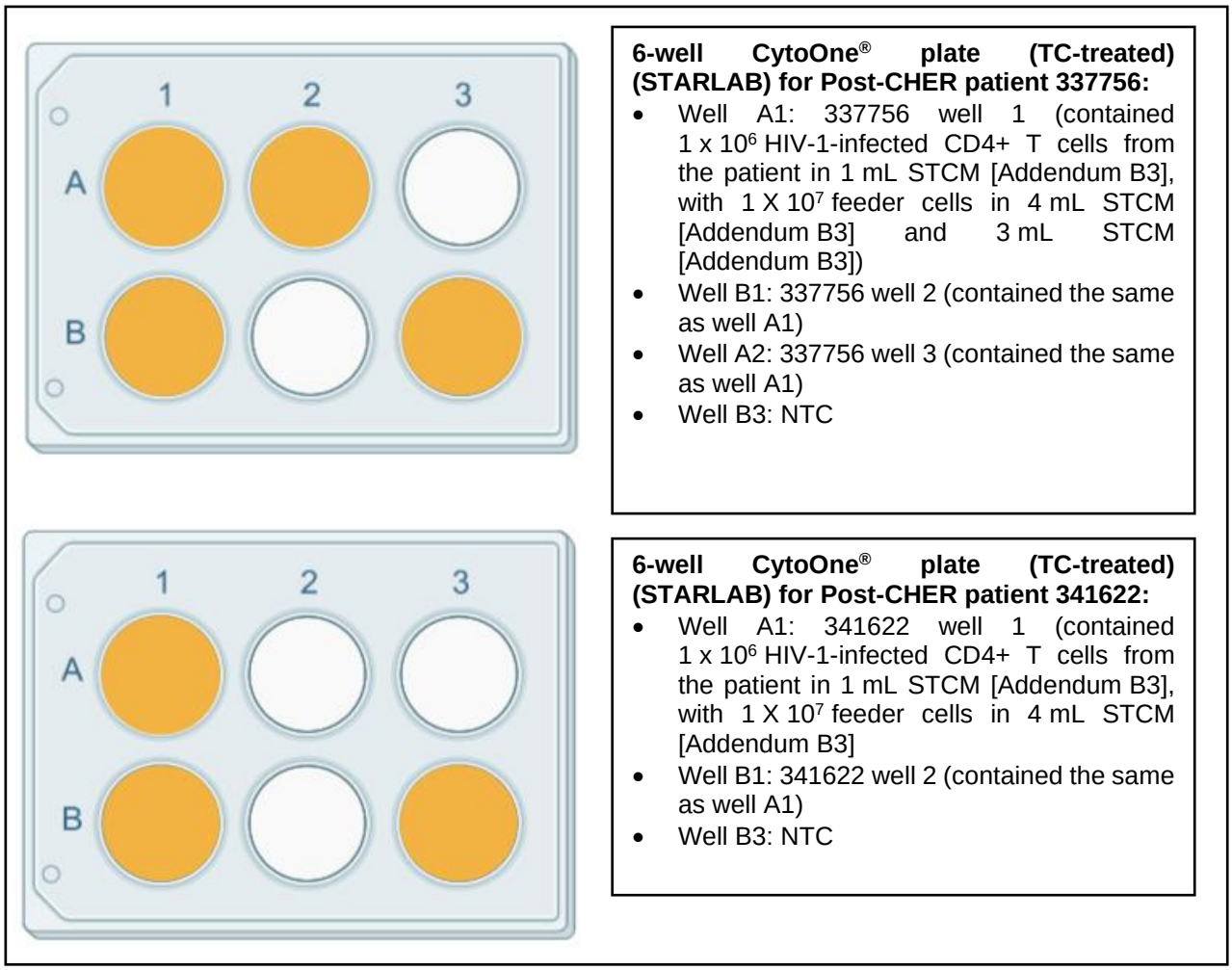
Figure 2.14: Plate layout and summary of what each 75 cm^2 flask contained for the 15 viraemic patients (adults) in the second experiment to evaluate the permissivity of the MOLT-4 CCR5+ cells

(Picture created with BioRender.com).

2.5.6 Second attempt at validating the VOA by using two recent viraemic patients from the Post-CHER cohort

A 28 day VOA as described earlier under Section 2.5.3 was performed to see whether replication-competent virus could be recovered from two Post-CHER recent viraemic, currently suppressed, children (patient 337756 and 341622). More information on these children can be found in Table 2.1. A layout of the plates for each patient can be seen in Figure 2.15. Supernatant was not only stored away for measuring exponential increases in HIV-1 RNA and infectious virus on days seven, 14, 21 and 28, but also on day five. The MOLT-4 CCR5+ cells were also assayed for the expression of the CCR5+ co-receptor and the CD4+ receptor via flow cytometry, as explained in Section 2.5.5, under subheading "Testing the MOLT-4 CCR5+ cells for expression of the CD4+ receptor and for the CCR5+ co-receptor" before starting the VOA. The percentage of total viable cells from each well from the 6 well plates (STARLAB) was determined by Trypan Blue exclusion as explained under Section 2.5.1 (the lower gate was set at 6 µm and the upper gate was set at 20 µm) on days nine, 16 and 23 to ensure that the MOLT-4 CCR5+ cells were viable enough for the expansion of HIV-1 throughout the experiment. Regarding isolation of total CD4+ T cells from patient PBMCs, the optimised protocol as explained under Section 2.5.5, subheading "Optimisation of total CD4+ T cell recovery from PBMC" was used. To be able to measure for exponential outgrowth of virus, HIV-1 RNA had to be extracted from 200 µL cell culture supernatant following the centrifugation protocol from the E.Z.N.A.® Viral RNA Kit (Omega Bio-Tek, Inc., Norcross, USA). The in-house RNA RT-qPCR assay was performed on the extracted RNA as described previously under sections 2.4.2 and 2.5.3 to measure for HIV-1 outgrowth over time. The data obtained from the standard curve of the RNA RT-qPCR assay from the patient samples at the different time points during the 28 day VOA, was used to plot a log-linear growth curve using R software version 4.0.3 (R Core Team, 2020). This was done by using the DNA signal (NRT control) and subtracting it from the RNA signal (RT reaction) obtained. Samples were also assayed for infectious virus by the p24 ELISA Kit (PerkinElmer®) to confirm the presence of infectious virus in wells that contained a positive

RNA signal with the RNA RT-qPCR assay and to compare sensitivity between the in-house RNA RT-qPCR assay and the p24 ELISA from PerkinElmer®.



6-well CytoOne® plate (TC-treated) (STARLAB) for Post-CHER patient 337756:

- Well A1: 337756 well 1 (contained 1 x 10^6 HIV-1-infected CD4+ T cells from the patient in 1 mL STCM [Addendum B3], with 1 X 10^7 feeder cells in 4 mL STCM [Addendum B3] and 3 mL STCM [Addendum B3])
- Well B1: 337756 well 2 (contained the same as well A1)
- Well A2: 337756 well 3 (contained the same as well A1)
- Well B3: NTC

6-well CytoOne® plate (TC-treated) (STARLAB) for Post-CHER patient 341622:

- Well A1: 341622 well 1 (contained 1 x 10^6 HIV-1-infected CD4+ T cells from the patient in 1 mL STCM [Addendum B3], with 1 X 10^7 feeder cells in 4 mL STCM [Addendum B3]
- Well B1: 341622 well 2 (contained the same as well A1)
- Well B3: NTC

Figure 2.15: Plate layout for the two Post-CHER children used in the VOA

(Figure was created in BioRender.com).

Chapter 3

Results

The results are presented in two main sections divided into further subsections. The first section highlights the results from the design and implementation of the HIV-1 RNA RT-qPCR assay that was used to measure inducible virus from cell culture supernatant extracted RNA, as well as outgrowth of virus (in the case of exponential increases in HIV-1 RNA). This section was divided into two subsections. The first section corresponds with objective four of this project, as the RNA RT-qPCR assay had to be implemented first before it could be used.

The second section focuses on the results obtained from the implementation of the VOA. Section two is divided into five subsections. The second section corresponds to objective one, two, three, four and five.

3.1 Design and Implementation of a Sensitive HIV-1 RNA RT-qPCR Assay

3.1.1 Preparation of the HIV-1 subtype C *integrase* standard for the HIV-1 RNA RT-qPCR assay

This first subsection shows the results obtained from the different steps involved in preparing the *integrase* RNA. Ten PCR products were produced from the linearised MJ4 (Ndung'u et al., 2001) plasmid. Gel-electrophoresis of the PCR products according to Figure 3.1 show that the DNA bands were each 418 bp in size, as expected.

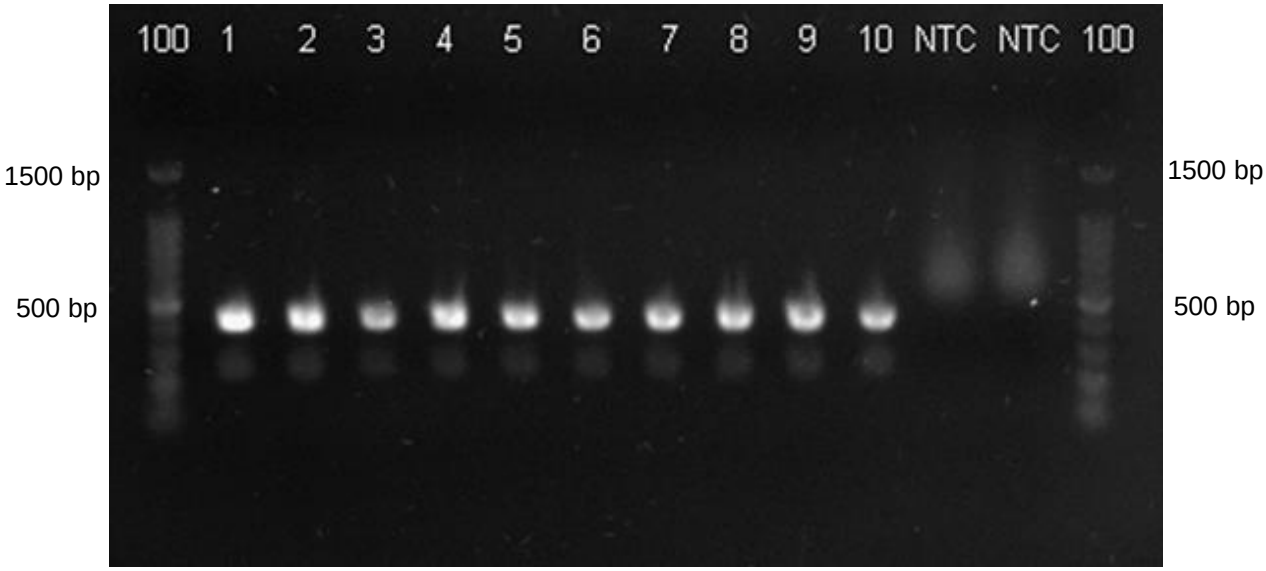


Figure 3.1: Gel-electrophoresis confirming the DNA band sizes of 418 bp after PCR from the MJ4 plasmid

100 represents the 100 bp DNA ladder (GeneDireX, Inc.), 1-10 represent the total number of PCR reactions and NTC represent the no template control which in this case consisted out of nuclease-free water (Qiagen).

The DNA was purified using the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel) by gel extracting the desired DNA bands. At the end of gel extraction, there was a total of four aliquots. The NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific) was used to determine the DNA purity, as well as the DNA concentration of each aliquot (Table 3.1). Aliquot number one was used for downstream experiments due to better purity achieved (the 260/230 ratio was closest to 1.8-2.2 for the DNA) in comparison with the other aliquots.

Table 3.1: NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific) readings for gel-extracted DNA aliquots 1-4

NanoDrop 1000 spectrophotometer Readings	**Aliquot**			
	1	**2**	**3**	**4**
260/280	1.73	1.88	1.52	1.73
260/230	1.07	0.82	0.47	0.35
ng/µL	122.40	129.60	121.10	73.35

The 260/280 ratio is known as the main measure of purity for DNA and RNA, for DNA a ratio of ~1.8 is usually accepted as pure. The 260/230 ratio is seen as a secondary measure of nucleic acid purity and it is often higher than the 260/280 ratio. A range of between 1.8-2.2 is generally accepted as pure for DNA and RNA. The concentration is shown in ng/µL (Thermo Fisher Scientific, 2010). Aliquot number one was chosen for the next experiment due to better purity achieved.

After gel-extraction purification, aliquot number one was cloned into vector pTZ57R/T (refer to Figure 2.3 in Section 2.4.1) downstream of the T7 promotor with the InsTAclone™ PCR Cloning kit (Thermo Fisher Scientific). First, the 418 bp gel-extracted DNA amplicon was ligated into the PTZ57R/T vector followed by the transformation of plasmid pTZ57R/T, containing the insert, into One Shot™ TOP10 Chemically Competent *E. coli* cells (Thermo Fisher Scientific). After the transformation step and allowing the bacteria to grow overnight, blue/white colony screening was performed so that the vector with the recombinant DNA (white colonies) could be selected. Five white colonies were selected. Three out of the five were scaled up to be able to generate sufficient recombinant DNA for downstream experiments while the other two colonies were used to generate glycerol stocks for long term storage. The plasmid DNA was isolated with the Thermo Scientific™ GeneJET™ Plasmid Midiprep Kit (Thermo Fisher Scientific). At the end of this purification step there were three aliquots. According to the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific), aliquot two obtained better DNA purity (the 260/230 ratio was closest to 1.8-2.2) and was therefore used in downstream experiments (Table 3.2).

Table 3.2: NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific) readings for aliquots 1-3 after plasmid DNA purification with the Thermo Scientific™ GeneJET™ Plasmid Midiprep Kit (Thermo Fisher Scientific)

NanoDrop 1000 spectrophotometer Readings	**Aliquot**		
	1	**2**	**3**
260/280	1.64	1.86	1.79
260/230	1.24	1.72	1.69
ng/µL	226.90	181.50	239.25

All three aliquots had good 260/280 ratios (close to 1.8). Although, aliquot two was chosen for the next experiment due to a better 260/230 ratio achieved.

The pure plasmid DNA was sequenced using a set of M13/pUC sequencing primers (Addendum A5) to ensure that the DNA amplicon was inserted into the vector. The FASTA sequences obtained were aligned to other biological sequences using Nucleotide BLAST on the National Center for Biotechnology Information (NCBI) website to determine whether the sequences were a match to the MJ4 plasmid. According to Nucleotide BLAST, the FASTA sequences had a 99.76% identity score to the HIV-1 clone MJ4 from Botswana (Accession number: AF321523.1). It was therefore concluded that this DNA insert was indeed a match.

The pure plasmid was linearised with the restriction enzyme EcoRI (Promega Corp.) in the MCS at 615 bp. For the linearisation reaction, a total of eight reactions were performed. After linearising the plasmid DNA, the product was purified with the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel). For this purification step, four aliquots from the linearisation reaction were pooled together. As such, there were two aliquots at the beginning of the purification step and two at the end. The purity of both the aliquots according to the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific) was acceptable as noted in Table 3.3.

Table 3.3: NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific) readings for aliquots 1 and 2 after purification of the linearised plasmid DNA with the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel)

NanoDrop 1000 spectrophotometer Readings	**Aliquot**	
	1	**2**
260/280	1.91	1.93
260/230	2.02	1.10
ng/µL	148.55	140.60

Both the aliquots achieved an acceptable purity. Both had a 260/280 ratio close to 1.8. Aliquot one had a better 260/230 ratio, but aliquot two's 260/230 ratio was still acceptable. Both aliquots were used for ethanol precipitation.

After purifying the linearised plasmid DNA with the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel), the DNA was concentrated through ethanol precipitation. The two aliquots' purity and concentration was measured using the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). Aliquot one obtained better DNA purity and had a higher DNA concentration compared to aliquot two (Table 3.4). Aliquot one was therefore used for *in vitro* RNA synthesis.

Table 3.4: NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific) readings for aliquots 1 and 2 after ethanol precipitation

NanoDrop 1000 spectrophotometer Readings	**Aliquot**	
	1	**2**
260/280	1.84	1.74
260/230	1.74	1.69
ng/µL	287.05	263.10

Both aliquots achieved acceptable purities, although aliquot one had a higher 260/230 ratio as well as a higher concentration and was therefore used for *in vitro* RNA synthesis.

In vitro RNA synthesis was performed using the Thermo Scientific™ TranscriptAid™ T7 High Yield Transcription Kit (Thermo Fisher Scientific). For the in vitro transcription reaction, a positive (Control DNA, 2.5 µg/µL) and negative control was included in the reaction with the DNA. The *in vitro* transcribed RNA was treated with DNase I (Thermo Fisher Scientific) to remove any residual template DNA. To use the RNA transcript as a standard in the RNA RT-qPCR assay, the RNA transcript together with the positive and negative controls were purified from any residual DNase with the PureLink® RNA Mini Kit (Invitrogen). To ensure that in vitro transcription was successful, and that the RNA transcript was pure, the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific) was used to determine the purity and concentration of the RNA.

Table 3.5: NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific) readings for the RNA standard, positive and negative controls after in vitro transcription, DNase treatment and purification

NanoDrop 1000 spectrophotometer Readings	**Sample**		
	RNA standard	**Positive Control**	**Negative Control**
260/280	2.20	2.22	2.30
260/230	2.45	2.38	0.74
ng/µL	796.00	1813.80	18.10

A 260/280 ratio of ~2.0 was seen as pure for RNA, a 260/230 ratio of between 1.8-2.2 was seen as pure for DNA and RNA and the concentration was in ng/µL (Thermo Fisher Scientific, 2010). Therefore, the RNA standard achieved an acceptable purity.

After *in vitro* RNA synthesis, an RNA transcript of 503 bp was expected. Figure 3.2 shows that the RNA transcript was 503 bp, the positive control was 2223 bp (according to the

Thermo Scientific™ TranscriptAid™ T7 High Yield Transcription Kit [Thermo Fisher Scientific]) and there was no RNA band present for the negative control.

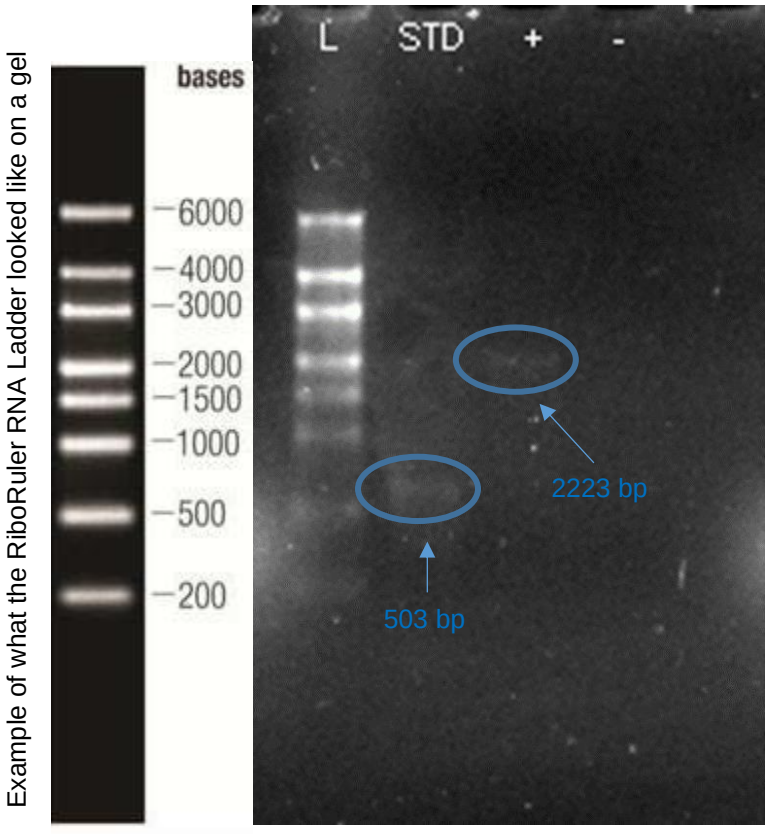


Figure 3.2: Analysis of RNA standard size via gel-electrophoresis

'L' represents the RiboRuler RNA Ladder, **'STD'** represents the newly synthesized RNA standard to be used in the RNA RT-qPCR assay at 503 bp, **'+'** represents the RNA positive control from the Thermo Scientific™ TranscriptAid™ T7 High Yield Transcription Kit (Thermo Fisher Scientific) at 2223 bp and **'-'** represents the NTC which in this case consisted out of nuclease-free water (Qiagen).

Finally, after determining that the RNA standard was the correct size of 503 bp, the RNA was quantified with the Qubit® 2.0 fluorometer (Thermo Fisher Scientific), using the Qubit® RNA HS Assay Kit (Thermo Fisher Scientific). A concentration of 59 ng/µL was obtained and this concentration was used to determine the RNA copy number of 21.73×10^{10} copies/mL with an online calculator, Endmemo (Endmemo, 2016). The RNA copy number was calculated by pasting in the length of the transcript (503 bp) and the weight of the transcript (59 ng, calculated by the Qubit® 2.0 fluorometer [Thermo Fisher Scientific]).

The RNA standard was diluted down from 21.73×10^{10} copies/mL to 1×10^{6} copies/µL for use in the RNA RT-qPCR assay. After the desired concentration of 1×10^{6} copies/µL was

reached, 96 single use aliquots were stored away at -80°C until use in the RNA RT-qPCR assay as the RNA standard.

3.1.2 Testing the RNA standard curves for DNA signal to evaluate DNase I efficiency

This subsection shows the results from testing the RNA standard for DNA signal after it was treated with DNase. Two RNA RT-qPCR experiments were performed with the RNA standard only to evaluate whether the DNase I was sufficient in removing any residual DNA signal. Both RT and NRT controls were included in the assays in triplicate for each dilution series of the RNA transcript. To determine what percentage of the RNA transcript attributed to DNA signal, the following test was performed: The average Ct was calculated for each of the RNA transcript dilutions (1 x 10^7 copies/10 µL down to 30 copies/10 µL) for both the RT and NRT reactions. After determining the delta Ct value for each dilution series of the RNA transcript, the average delta Ct (difference in Ct for the combined RNA + DNA and DNA alone) was calculated to be 7.13 for experiment one and 6.57 for experiment two (Table 3.6). The optimal gradient for 100% efficiency is 3.32, however, the observed gradients for the two experiments were -3.31 (Figure 3.3) and -3.53 (Figure 3.4) respectively. Following this, the fold difference of the RNA + DNA: DNA signal in log scale was calculated from the Ct difference and gradients. The fold difference of the RNA + DNA: DNA signal for experiment one and two was 142.59 and 72.64 receptively (Table 3.6). Transforming these log values by taking the anti-log we found that the DNA contribution of the total signal was 0.7% and 1.4% in the respective experiments (Table 3.6). Therefore, since the percentage of DNA that contributed to the signal detected by the RNA transcript was so small, it was seen as an acceptable purity for the RNA standard. For quality control purposes, when performing the VOA on patient samples, a NRT control would be included for the HIV-1-infected samples when measuring for outgrowth of infectious HIV-1.

Table 3.6: Determining what percentages of the RNA Transcript attributed to DNA signal

Number RNA RT-qPCR assay performed	Average Ct differences between the RT signal versus the NRT signal	Observed assay gradient for standard curve[a]	RT signal: NRT signal	Percentage of RNA transcript that attributed to DNA signal
1	7.13	-3.31	142.59	0.7%
2	6.57	-3.53	72.64	1.4%

[a]A good qPCR reaction should have an efficiency of between 90% and 110%, which corresponds to an assay gradient of -3.58 and -3.10 (according to LifeTechnologies™).

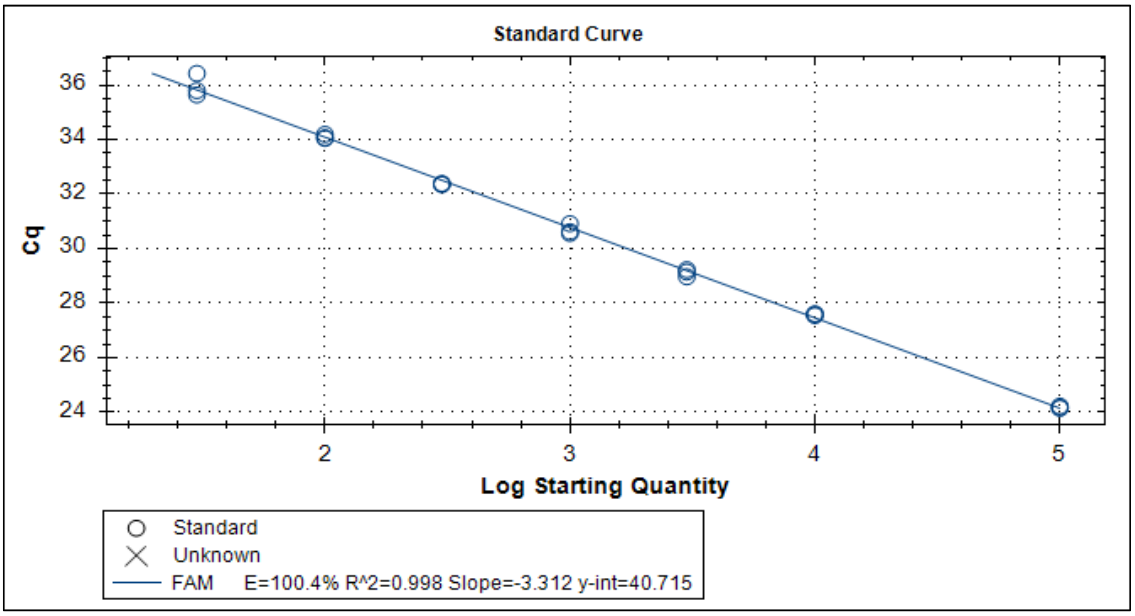


Figure 3.3: Standard curve of experiment one in testing the RNA transcript for DNA signal

The efficiency (E) of this reaction was good as it was between 90% and a 110%. The correlation coefficient (R^2) was considered acceptable as it was close to 1.

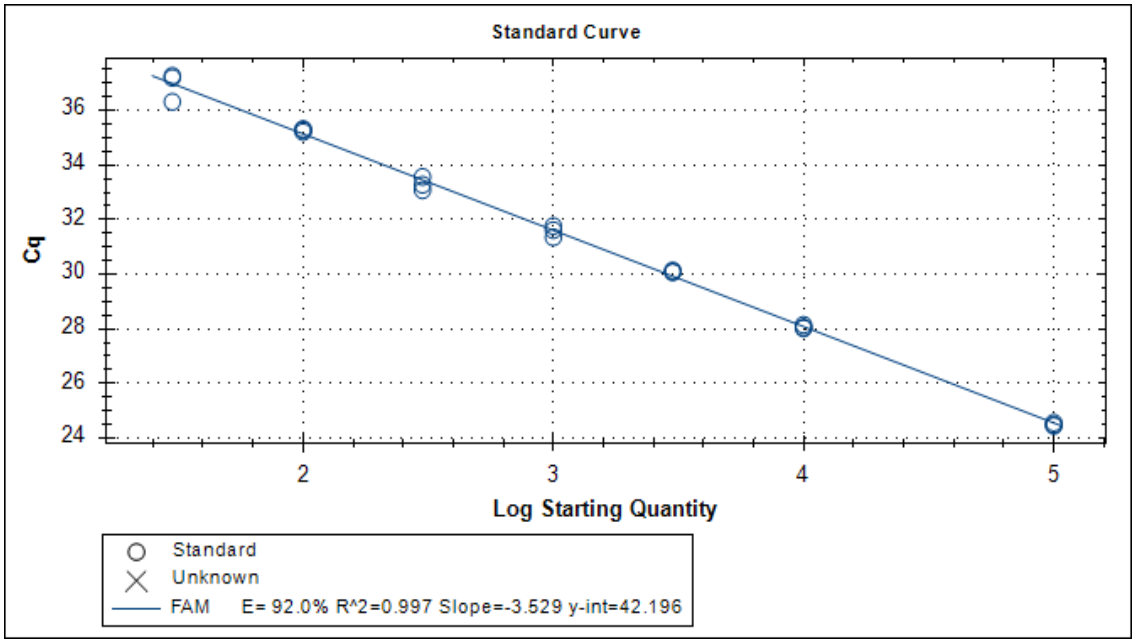


Figure 3.4: Standard curve of experiment two in testing the RNA transcript for DNA signal

The efficiency (E) for this experiment was between 90% and a 110% and was therefore considered acceptable. This experiment's correlation coefficient (R^2) was close to 1 and was therefore also considered acceptable.

3.2 Implementation of the VOA

3.2.1 Results of evaluating the efficiency of the TCGF

The first subsection focuses on the results obtained from evaluating the efficiency of the VOA reagent, TCGF, after it was prepared in-house. This was done by using the MTS Cell Proliferation Kit (Colorimetric)(Abcam®). The absorbance from each well on the 96-well TC-treated microplate (Corning® Incorporated) was measured from the formazon that was produced by viable cells based on the reduction of MTS tetrazolium compound after addition of the MTS reagent (MTS Cell Proliferation Assay Kit [Colorimetric] [Abcam®]). The OD reading of 1.98 was the highest absorbance at 492 nm, therefore 2% was the optimal concentration of TCGF (in-house) to add to the cell culture media used in the VOA for maximal cell proliferation (Figure 3.5).

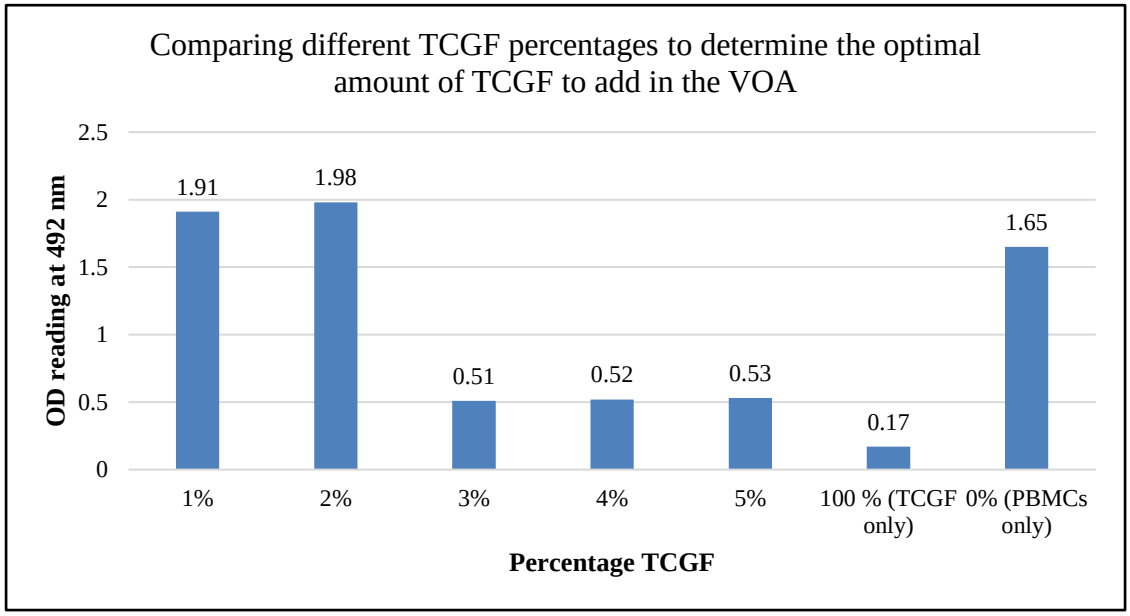


Figure 3.5: Comparison of different TCGF percentages in order to determine the optimal amount to add in the VOA

Different percentages of TCGF was compared to determine the optimal concentration to add to cell culture media for use in the VOA. The percentage of TCGF that produced the highest OD reading at 492 nm would be the best concentration to use. Therefore, 2% TCGF was the optimal concentration to use in the VOA.

3.2.2 Microscopic pictures of the MOLT-4 CCR5+ cell line in culture

Figure 3.6 shows two light microscopic pictures taken of the MOLT-4 CCR5+ cell line. The cells were examined under an inverted ZEISS Primovert light microscope (Carl Zeiss AG)

and photos were taken using the Axiocam ERc 5s camera (Carl Zeiss AG). The photographs were analysed with ZEN Blue Edition Version 3.2.0 (Carl Zeiss AG).

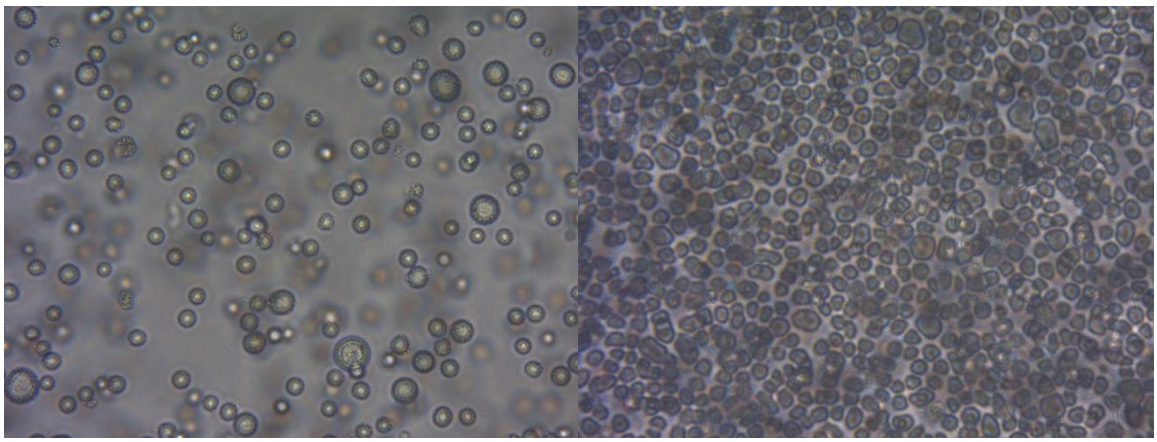


Figure 3.6: Light microscopic pictures of the MOLT-4 CCR5+ cell line at two different time points in culture

The picture on the left shows the cells one day after they were passaged 1:10. The picture on the right shows the cells when they reached confluency just before they were passaged. The cells were observed microscopically at X200.

3.2.3 Results obtained from the first validation attempt using fresh EDTA blood from two viraemic patients

The VOA was performed over 35 days using fresh EDTA blood obtained from two viraemic patients (Table 2.2) as described previously under Section 2.5.4 in the Materials and Methods Chapter (Chapter 2). The experiment was done to attempt to validate the VOA.

There was no infectious virus detectable, as measured by the HIV-1 p24 core antigen from cell culture supernatant with the HIV-1 p24 ELISA kit (PerkinElmer®) on days seven, 14, 21, 28 and 35, as none of the sample wells turned yellow after the incubation step with OPD. The percentage viability as determined by Trypan Blue exclusion for the patient wells was low on days 14, 21 and 35. It ranged between 10% and 41%, this could be due to not splitting the cultures on the indicated days as described in the VOA protocol in Section 2.5.3. According to Table 3.7, no increase of HIV-1 RNA was observed for any of the two viraemic HIV-1-infected patients over time; therefore, there was no HIV-1 replication-competent virus present.

Since this validation experiment was not successful, troubleshooting to optimise the VOA and to increase chances of recovering inducible and/or infectious virus was performed.

Table 3.7: Viral load results from the VOA wells of each viraemic patient to test for increased levels of HIV-1 RNA

Patient ID And Well[a] of 6-well plate (STARLAB)	**Day[b]**	**Viral Load (copies/mL)[c]**
Patient 1 (Viraemic Adult), Well A1	7	Target Not Detected
	14	<20
	21	Target Not Detected
	28	Target Not Detected
	35	Target Not Detected
Patient 2 (Viraemic Child), Well A1	7	Target Not Detected
	14	Target Not Detected
	21	Target Not Detected
	28	Target Not Detected
	35	<20
Patient 2 (Viraemic Child), Well B1	7	179
	14	98
	21	35
	28	<20
	35	<20

[a]The wells were annotated according to the amount of wells each patient had as seen in Figure 2.12 under Chapter 2. [b]The day of the VOA from which the HIV-1 RNA viral load was measured from. [c]The viral load in copies/mL as measured by the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test, v2.0 (Roche Holding AG).

3.2.4 Results from troubleshooting

3.2.4.1 Optimisation of total CD4+ T cell recovery from PBMC

A summary of the results obtained from optimising the 5 mL protocol from the EasySep™ Human CD4+ T Cell Isolation Kit (STEMCELL™ Technologies Inc.) with the EasyEights™ Magnet (STEMCELL™ Technologies Inc.) to recover maximal total CD4+ T cells from PBMCs can be seen in Figure 3.7. In experiment one, 42 x 10^6 PBMCs were isolated and from this a total of 1.96 x 10^6 CD4+ T cells were recovered which gave a percentage of 4.67% CD4+ T cells recovered from the total PBMCs. Experiment two was able to recover 15.37% of total CD4+ T cells from PBMCs. By separating the isolated unwanted cells twice with immunomagnetic negative selection and by combining the enriched cell suspension from this tube to the enriched cell suspension from the first separation, optimal CD4+ T cells

were recovered. Therefore, experiment three gave the highest CD4+ T cell recovery of 23.96% and was therefore chosen as the protocol to use in isolating total CD4+ T cells from PBMCs going forward.

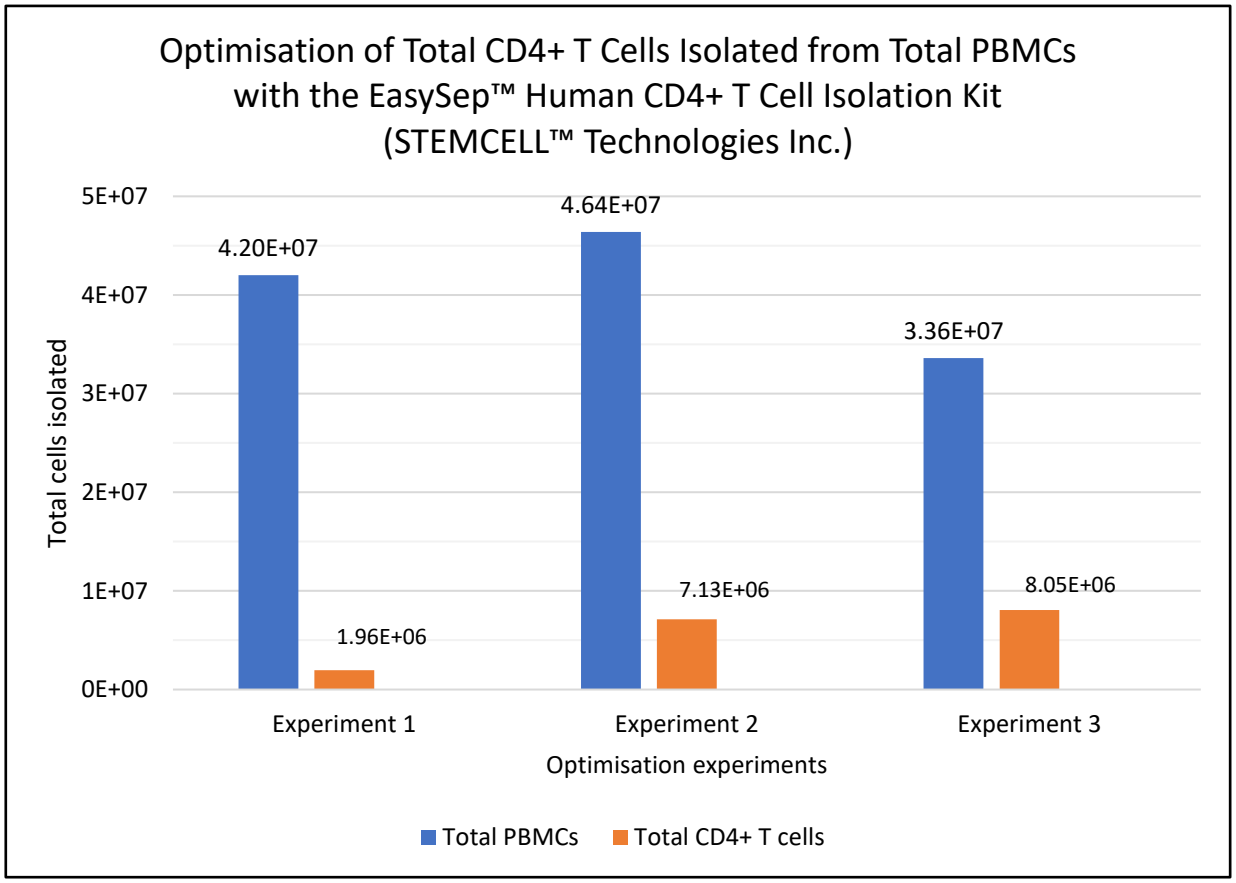


Figure 3.7: Results of the three optimisation experiments to increase the total CD4+ T cells isolated from total PBMCs

3.2.4.2 Testing the MOLT-4 CCR5+ cells for expression of the CD4+ receptor and the CCR5+ co-receptor

The optimal volume of each monoclonal antibody, PE CD195 (CCR5+) (Biolegend®) and BB515 Mouse Anti-Human CD4+ (Clone RPA-T4) (BD Biosciences), to use with MOLT-4 CCR5+ cells were determined via titrations. After comparing different volumes of the antibodies, it was concluded that the optimal staining volume for the PE CCR5+ antibody (Biolegend®) was 2.5 µL and for the BB515 CD4+ antibody (BD Biosciences) it was 5 µL, as shown in Figure 3.8, as there was a clear shift from the negatively stained to the positively stained MOLT-4 CCR5+ cells.

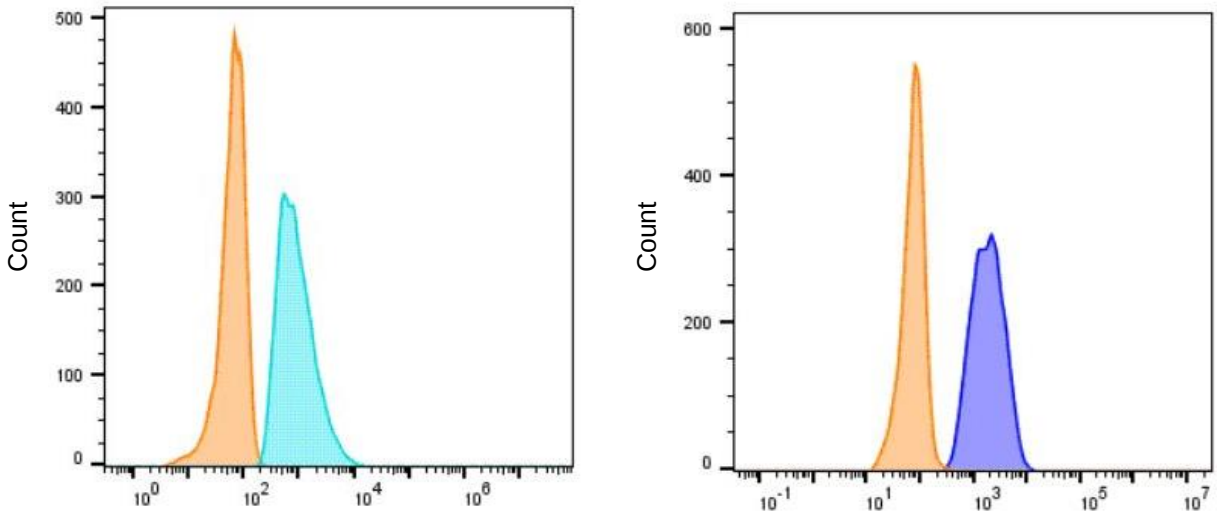


Figure 3.8: MOLT-4 CCR5+ cells stained with the PE CCR5+ antibody (Biolegend®) and with the BB515 CD4+ antibody (BD Biosciences)

The graph on the left demonstrates the MOLT-4 CCR5+ cells stained with 2.5 µL PE CCR5+ antibody (Biolegend®). There was a clear shift in the unstained population (shown in orange) to the stained population (shown in light blue). The graph on the right demonstrates the MOLT-4 CCR5+ cells stained with 5 µL BB515 CD4+ antibody (BD Biosciences). Once again, a clear shift can be seen from the unstained population, shown in orange, to the stained population, shown in dark blue.

The optimum PMT voltage for both the PE CD195 (CCR5+) monoclonal antibody (Biolegend®) as well as for the BB515 Mouse Anti-Human CD4+ (Clone RPA-T4) monoclonal antibody (BD Biosciences) were determined via voltration. As observed in Figure 3.9, Figure 3.10, Figure 3.11 and Figure 3.12 the point on the figure where a plateau was reached is where there would be optimal separation between the positive and negative populations and therefore this would be the optimal PMT voltage for each antibody. The optimal PMT voltage as determined by the separation index formula for the CCR5+ monoclonal antibody (Biolegend®) was 500 V (Figure 3.9) and for the BB515 CD4+ monoclonal antibody (BD Biosciences) it was also 500 V (Figure 3.11). The second formula used (stain index), obtained the same results for both antibodies as seen in Figure 3.10 and Figure 3.12.

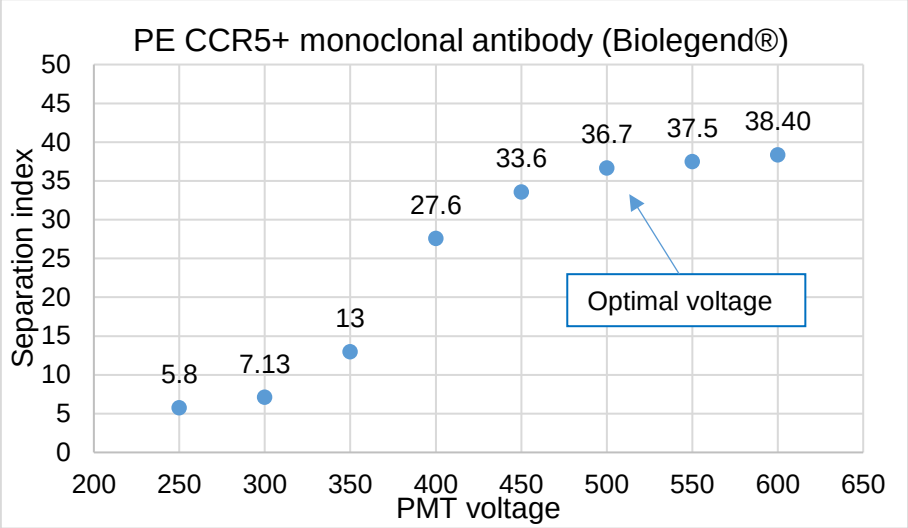


Figure 3.9: Voltration results of the CCR5+ monoclonal antibody (Biolegend®) using the separation index formula

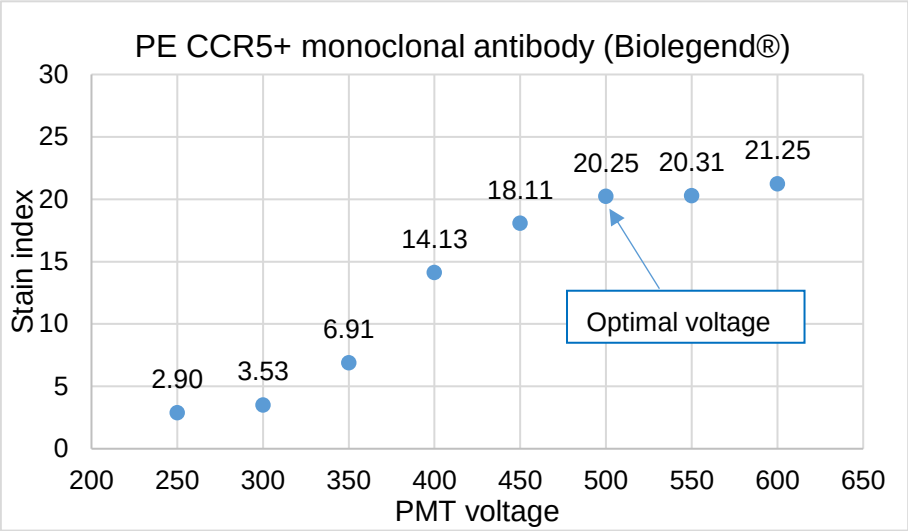


Figure 3.10: Voltration results of the CCR5+ monoclonal antibody (Biolegend®) using the stain index formula

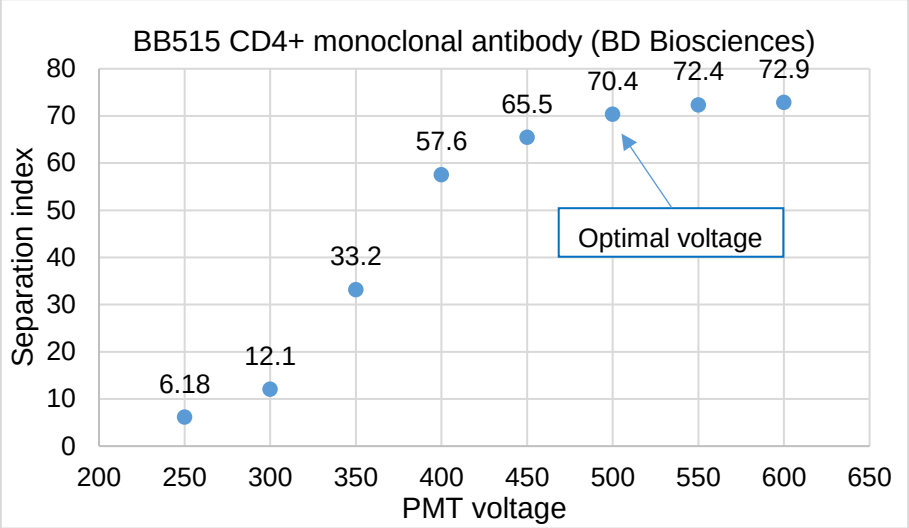


Figure 3.11: Voltration results of the BB515 CD4+ monoclonal antibody (BD Biosciences) using the separation index formula

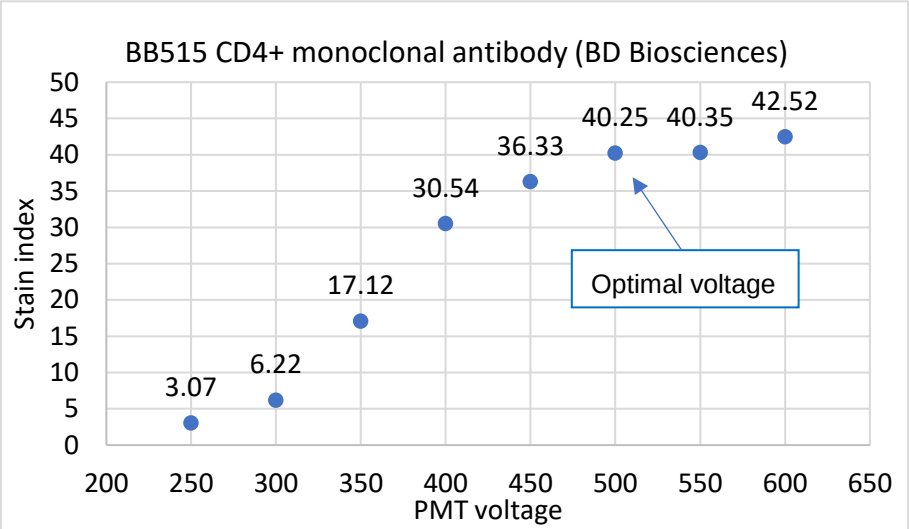


Figure 3.12: Voltration results of the BB515 CD4+ monoclonal antibody (BD Biosciences) using the stain index formula

3.2.4.3 Testing to determine if the MOLT-4 CCR5+ cells were permissive to HIV-1 infection

Two different experiments were performed to determine whether the MOLT-4 CCR5+ cell line was permissive to HIV-1 infection. In the first experiment the MOLT-4CCR5+ cells were infected with cell-free HIV-1 from frozen aliquots from two viraemic (viral load >500 copies/mL) patient samples from the Post-CHER cohort (Table 2.3). The viability of the MOLT-4 CCR5+ cells from the patient wells from the 6-well plates (STARLAB) on days seven, 14, 21 and 28 was good, it ranged from 65% to 85%. Infectivity of the MOLT-

4 CCR5+ cells were assayed with the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test, v2.0 (Roche Holding AG) on days seven, 14, 21 and 28. As observed from Table 3.8, there was no exponential increase in HIV-1 RNA for either of the patients. Cell culture supernatant was also assayed for p24 antigen with the p24 ELISA kit (PerkinElmer®), but no positive results were obtained.

It was inconclusive whether the MOLT-4 CCR5+ cells were permissive or not after this experiment, as several factors could have influenced the results: a validated free-virus culture method was not implemented and conditions for cell culture inoculation may have been sub-optimal. Moreover, it was not known if, despite the high viral loads, the viruses retained infectivity as infectivity may be reduced by freeze-thaw cycles.

Table 3.8: Viral Load results from the two Post-CHER viraemic frozen plasma samples to test for HIV-1 outgrowth to determine whether the MOLT-4 CCR5+ cells were permissive to HIV-1 infection

Patient ID and visit number[a]	**Day[b]**	**Viral load (copies/mL)[c]**
334846 V6	7	1338
	14	277
	21	96
	28	<20
360582 V7	7	100
	14	Target Not Detected
	21	<20
	28	<20

[a] The visit number refers to the patient's follow-up visits where clinical assessments were done. Patient 334846 was 12 (turning 13) at visit number six and patient 360582 was 13 (turning 14) at visit number seven. [b] The day of the VOA from which HIV-1 RNA was measured from. [c] The viral load in copies/mL as measured by the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test, v2.0 (Roche Holding AG).

In the second experiment to test the permissivity of the MOLT-4 CCR5+ cells, fresh plasma from 15 viraemic adults was obtained (Table 2.4). The in-house RNA RT-qPCR assay was used to measure for outgrowth of HIV-1 over time from days three, seven and 10 from RNA extracted from cell culture supernatant. Figure 3.13 shows the log amplification curve of the dilution series of the RNA standard used in the in-house RNA RT-qPCR assay and Figure 3.14 shows the standard curve for the dilution series of the RNA standard. The baseline threshold was set manually at 62.70. Figure 3.16 shows the log amplification curve of days

three, seven and 10 of flask A that contained pooled plasma of the following viraemic adults (Figure 2.14): 003, 004, 005, 006 and 011. This log amplification curve shows that there was no HIV-1 outgrowth, as the curves from day seven picked up later than day three and no HIV-1 cell-free RNA was detected on day 10. The log amplification of Flask B (Figure 2.14) is depicted in Figure 3.15. Flask B contained the pooled plasma of viraemic adults 009, 010, 012, 013, 014 and 015. There was no HIV-1 outgrowth present in flask B as well as the RNA curves picked up at later cycles on day seven and again on day 10. Figure 3.17 shows the log amplification curve for the pooled plasma from viraemic adults 001 and 002 from well A1 on the 6-well plate (STARLAB) (Figure 2.14). Interestingly, there was an increase in HIV-1 RNA on day 10, although only one curve picked up on day 10 during the RNA RT-qPCR assay (each sample at each time point was assayed in duplicate for HIV-1 RNA). The log amplification curve for the pooled plasma from viraemic adults 007 and 008 (Figure 2.14) from well B1 from the 6-well plate (STARLAB) can be seen in Figure 3.18. There was no HIV-1 outgrowth detected in well B1. The NTC well (well B3 from the 6-well plate [STARLAB]) (Figure 2.14) was clear of any RNA (the RT reaction of the RNA RT-qPCR assay) and DNA (the NRT reaction from the RNA RT-qPCR assay). Both 75 cm^2 cell culture flasks (Thermo Fisher Scientific) and both wells from the 6-well plate (STARLAB) were clear of any DNA signal (the NRT reaction from the RNA RT-qPCR assay). The MOLT-4 CCR5+ cells from the patient wells (wells A1 and B1) on the 6-well plate (STARLAB) and the two 75 cm^2 cell culture flasks (Thermo Fisher Scientific) had poor viability throughout this experiment (it ranged from 8% to 40%).

After troubleshooting the validation of the VOA, it was noted that at the first attempt of validating the VOA and also in the two permissivity experiments with the cell-free plasma the recombinant IL-2 (Thermo Fisher Scientific) was too diluted when it was added to the STCM (this was an operator error). It was added at a concentration of 10 U/mL instead of 100 U/mL. This was important as the addition of IL-2 in combination with PMA (that was part of the in-house prepared TCGF), PHA and irradiated feeder cells play an important role in the stimulation of T cells in order to reverse latency (Massanella & Richman, 2016).

Since permissivity could not be proved from these two experiments with cell-free plasma, it was decided to validate the VOA again using the co-culture method (with HIV-1-infected CD4+ T cells from patients) as the expansion cells for HIV-1 (the MOLT-4 CCR5+ cell line) did indeed express the CD4+ receptors and the CCR5+ co-receptors as seen before.

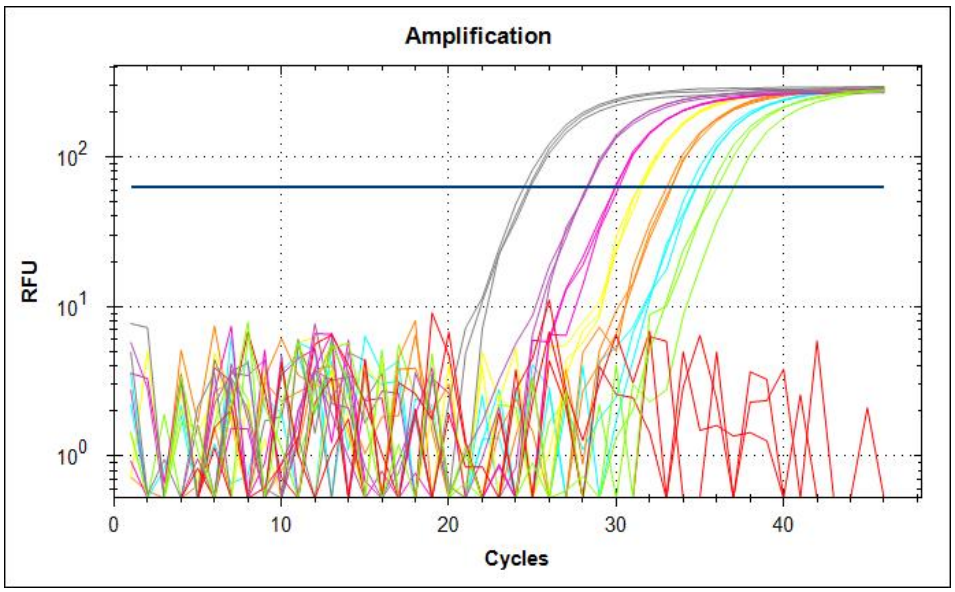


Figure 3.13: Log amplification curve of the RNA standard dilution curves in the second experiment of testing the permissivity of the MOLT-4 CCR5+ cells with 15 viraemic adults

The colour scheme for the log amplification RNA standard curves were as follows: light green represented 30 copies/well, light blue represented a 100 copies/well, orange represented 300 copies/well, yellow represented a 1000 copies/well, pink represented 3000 copies/well, purple represented 10 000 copies/well and grey represented a 100 000 copies/well. Each standard curve was assayed in triplicate. All three of the NTC wells were clear (represented in red). RFU: relative fluorescence units.

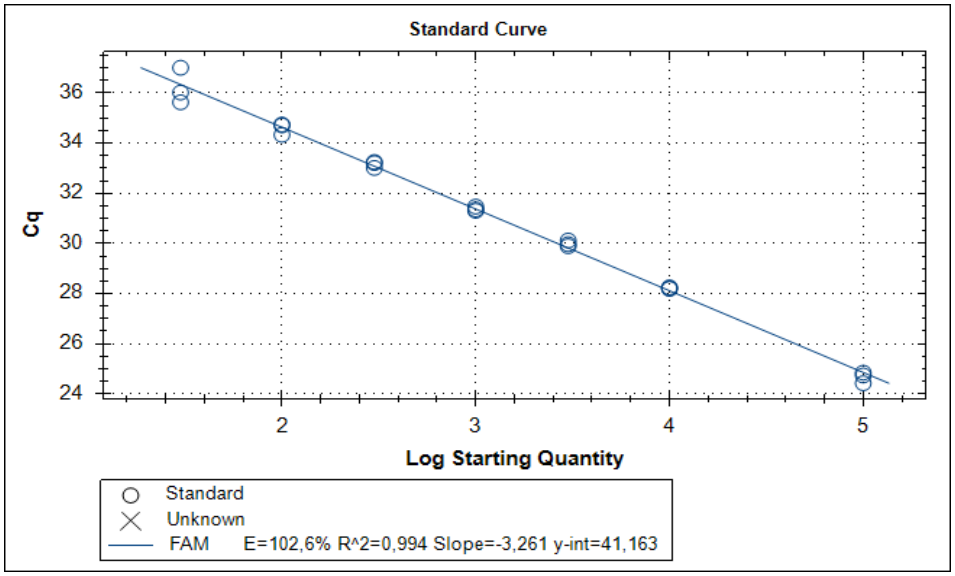


Figure 3.14: Standard curve for the RNA standard dilution curves in the second experiment of testing the permissivity of the MOLT-4 CCR5+ cells with 15 viraemic adults

'E': This qPCR had a good efficiency of 102.6% (within the range of 90%-110%).
'R^2': The correlation coefficient was good, because it was close to 1.

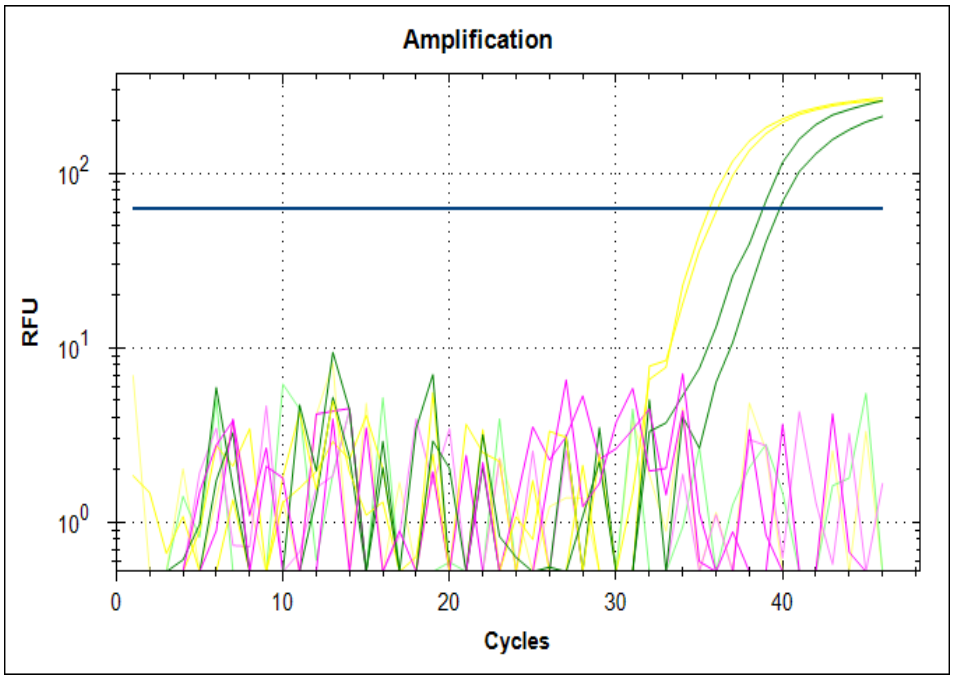


Figure 3.16: Log amplification curve for Flask A (Figure 2.14) in testing the permissivity of the MOLT-4 CCR5+ cells with 15 viraemic adults

The different colours represent the different time points during the experiment: Yellow is day three of the experiment, green is day seven and pink is day 10. Each sample was assayed in duplicate.

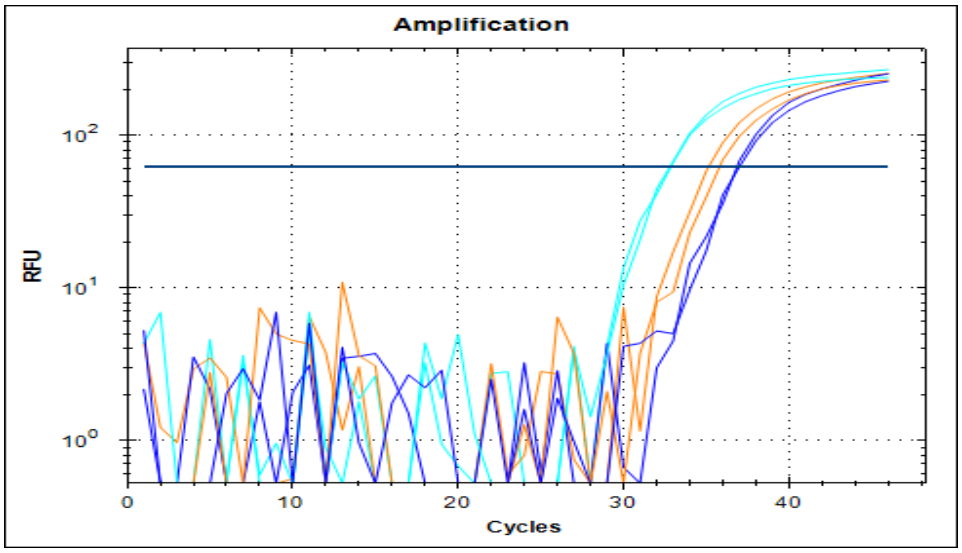


Figure 3.15: Log amplification curve for Flask B (Figure 2.14) in testing the permissivity of the MOLT-4 CCR5+ cells with 15 viraemic adults

The different colours represent the different time points during the experiment: Light blue is day three of the experiment, orange is day seven and dark blue is day 10. Each sample was assayed in duplicate.

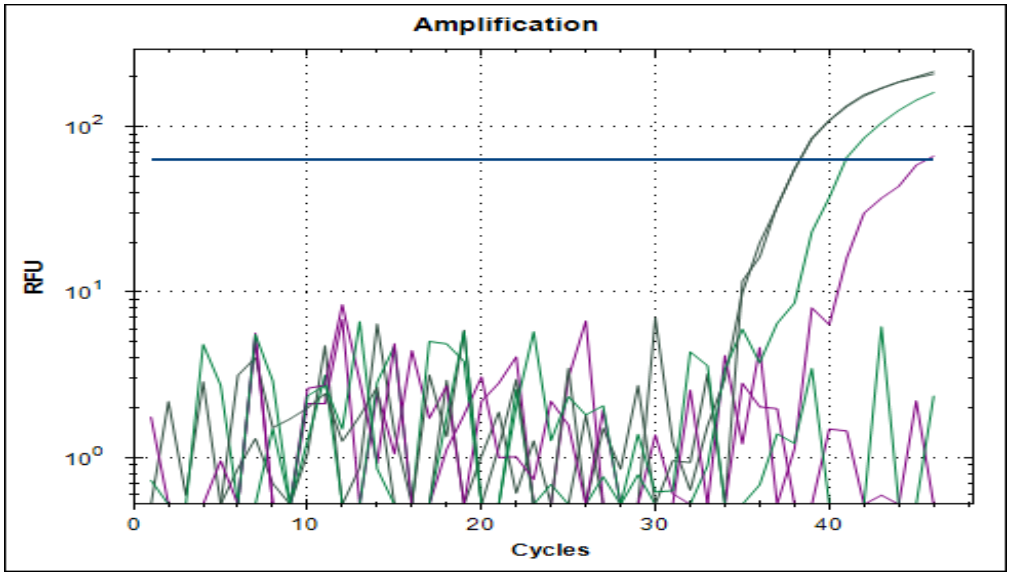


Figure 3.17: Log amplification curve for well A1 of the 6-well plate (STARLAB) (Figure 2.14) in testing the permissivity of the MOLT-4 CCR5+ cells with 15 viraemic adults

The different colours represent the different time points during the experiment: Dark green is day three of the experiment, purple is day seven and light green is day 10. Each sample was assayed in duplicate in the RNA RT-qPCR assay, for days seven and 10 only one of the replicates picked up out of two.

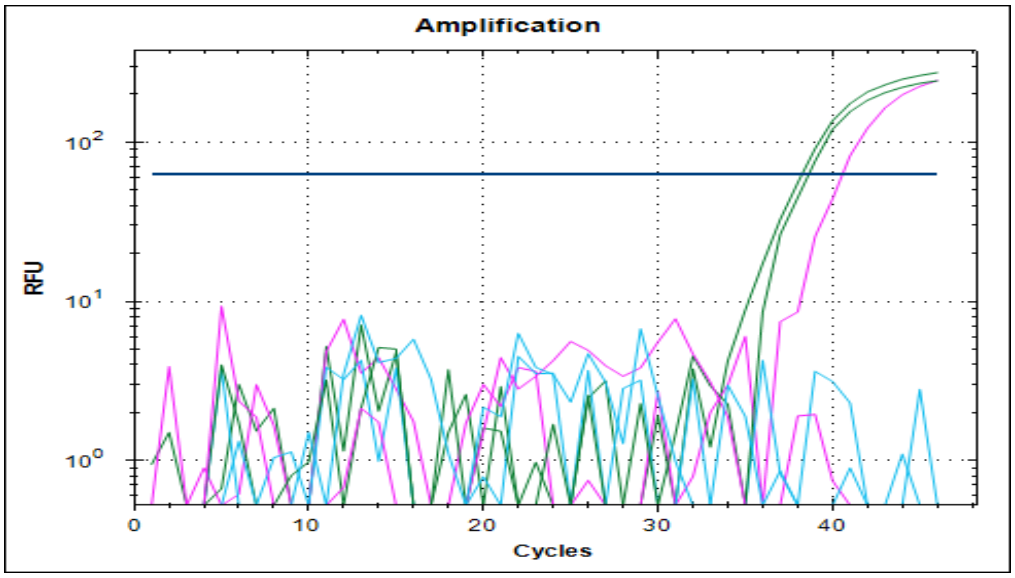


Figure 3.18: Log amplification curve for well B1 of the 6-well plate (STARLAB) (Figure 2.14) in testing the permissivity of the MOLT-4 CCR5+ cells with 15 viraemic adults

The different colours represent the different time points during the experiment: Dark green is day three of the experiment, pink is day seven and light blue is day 10. Each sample was assayed in duplicate in the RNA RT-qPCR assay, for day seven only one of the replicates picked up out of two and on day 10 no RNA was detected.

3.2.5 Results of the second validation process of the VOA

A 28-day VOA was performed with two Post-CHER recent viraemic, suppressed children. More information on these children can be found in Table 2.1. Information on the first patient's (337756) viral load and iCAD data can be seen in Figure 3.19 and Figure 3.20 respectively. Figure 3.21 and Figure 3.22 show information on the second patient's (341622) viral load and iCAD data, respectively. One of the children also had high diversity according to the CAD-SGS assay. A neighbour-joining phylogenetic tree was constructed for patient 337756, at time point visit four (sample date 15/12/2016), to determine the virus' diversity (Figure 3.23). The diversity of the HIV-1 virus was calculated in Mega 5.0 using the average pairwise distance (APD) calculation. This patient had a high HIV-1 DNA diversity of 0.015879 (1.6%). Flow cytometric analysis showed that the MOLT-4 CCR5+ cells constitutively expressed both the CD4+ receptor and the CCR5+ co-receptor. Most of the total cells plated in each well of the 6-well plates (STARLAB) were viable as determined by Trypan Blue exclusion (it ranged from 79% to 95%) during the 28-day VOA.

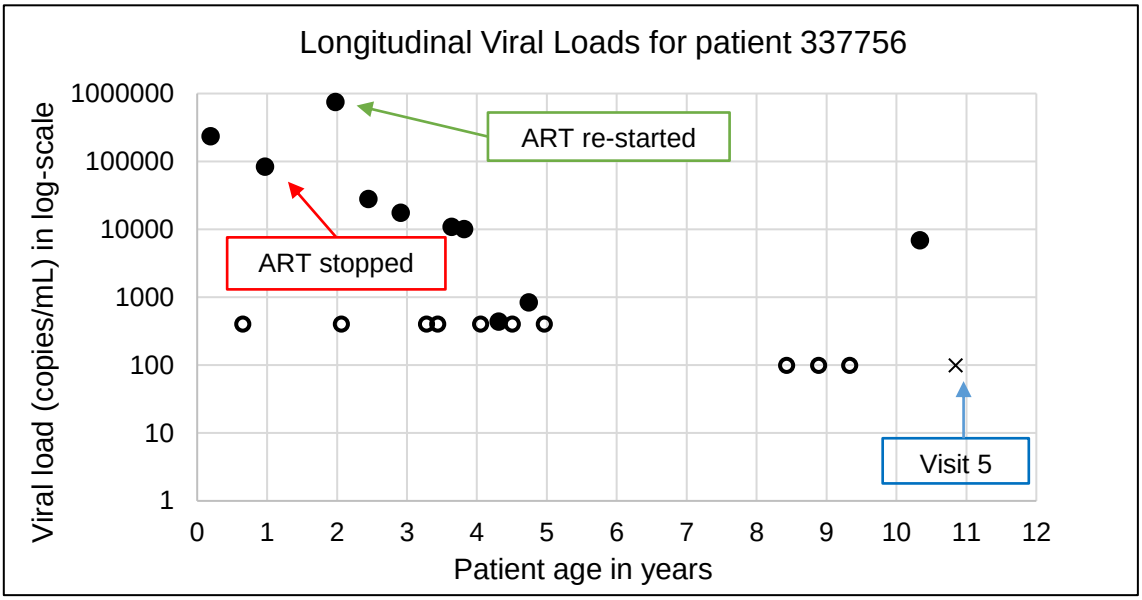


Figure 3.19: Longitudinal viral loads (copies/mL) for Post-CHER patient 337756

Viral loads in copies/mL were taken routinely for the Post-CHER patient at different time points. Open circles indicate viral loads that were suppressed according to the viral load assay used at that time point and full circles indicate unsuppressed viral loads. For this study's purposes, the patient had to be virologically suppressed at the time point chosen, but also had to have a recent episode of viraemia. Therefore, the visit five sample was chosen for Post-CHER patient 337756 (indicated with a cross).

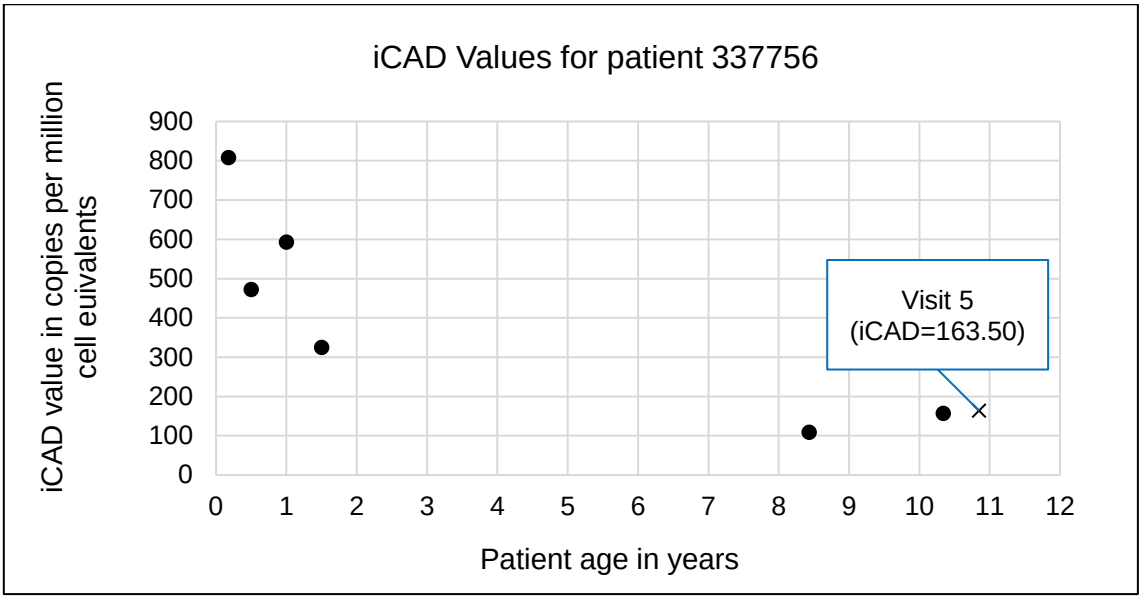


Figure 3.20: iCAD values at different time points for Post-CHER patient 337756

The iCAD values (copies per million cell equivalents) were determined with the quantitative iCAD assay at certain time points (visit dates). The visit five sample was chosen for this Post-CHER patient (337756) as the viral load (copies/mL) was suppressed at this time point with a recent episode of viraemia as was the requirements of this study (see Figure 3.19) and this sample had an iCAD value of >50 copies per million PBMCs.

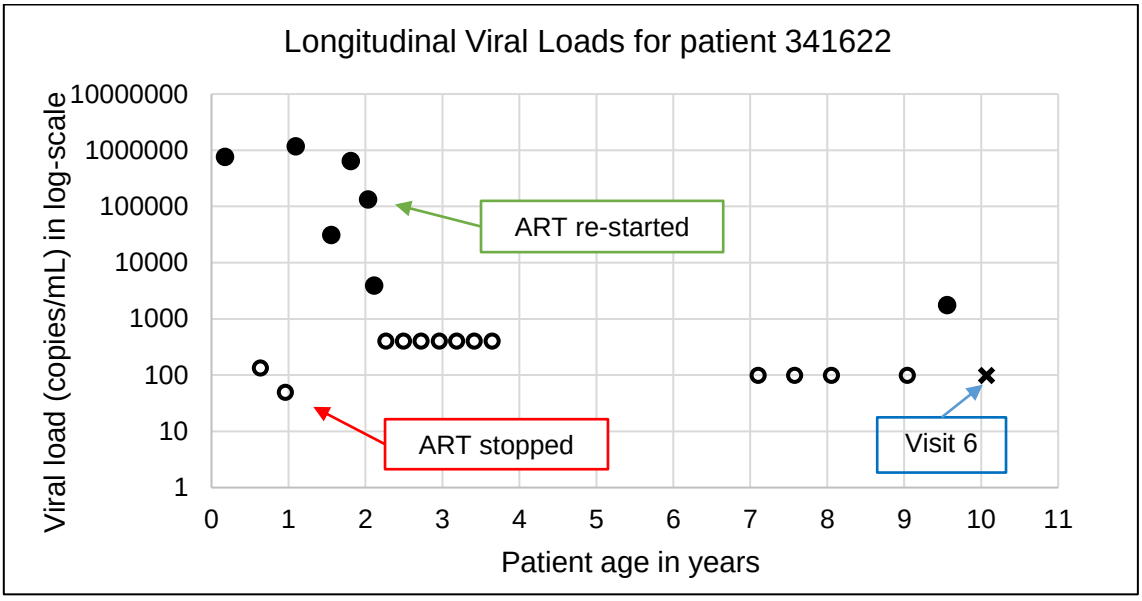


Figure 3.21: Longitudinal viral loads (copies/mL) for Post-CHER patient 341622

Viral loads in copies/mL were taken routinely for the Post-CHER patient at different time points. Open circles indicate viral loads at specific time points that were suppressed according to the viral load assay used at that time point. Closed circles indicate unsuppressed viral loads. For this study's purposes, the patient had to be virologically suppressed at the time point chosen, but also had to have a recent episode of viraemia. Therefore, the visit six sample was chosen for Post-CHER patient 341622 (indicated with a cross).

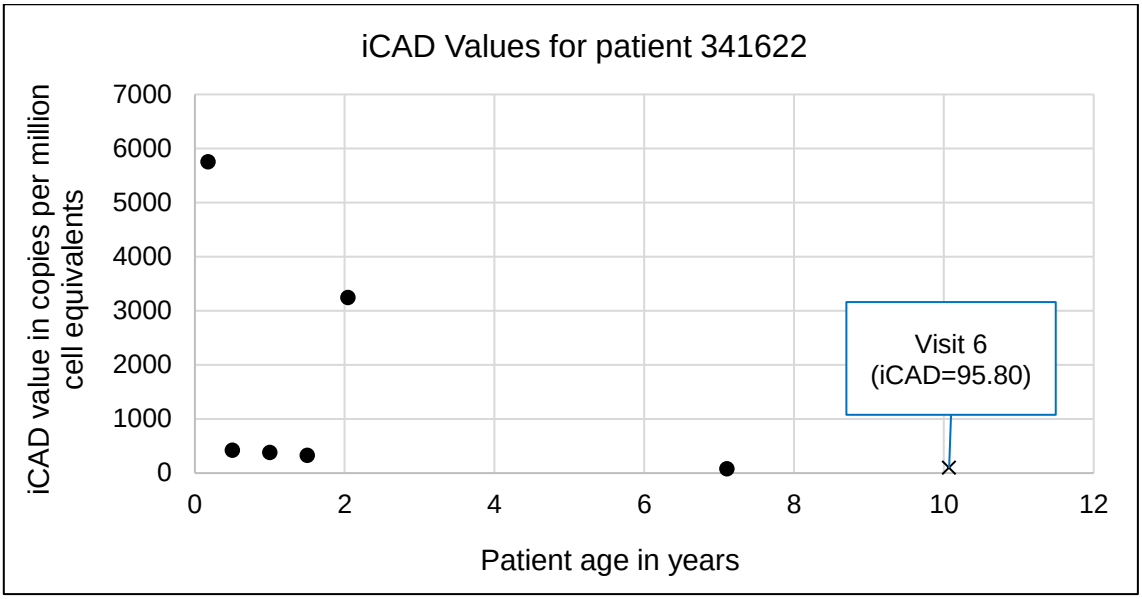


Figure 3.22: iCAD values at different time point for Post-CHER patient 341622

The iCAD values (copies per million cell equivalents) were determined with the quantitative iCAD assay at certain time points (visit dates). The visit six sample was chosen for this Post-CHER patient (341622) as the viral load (copies/mL) was suppressed at this time point with a recent episode of viraemia as was the requirements of this study (see Figure 3.21) and this sample had an iCAD value of >50 copies per million cell equivalents.

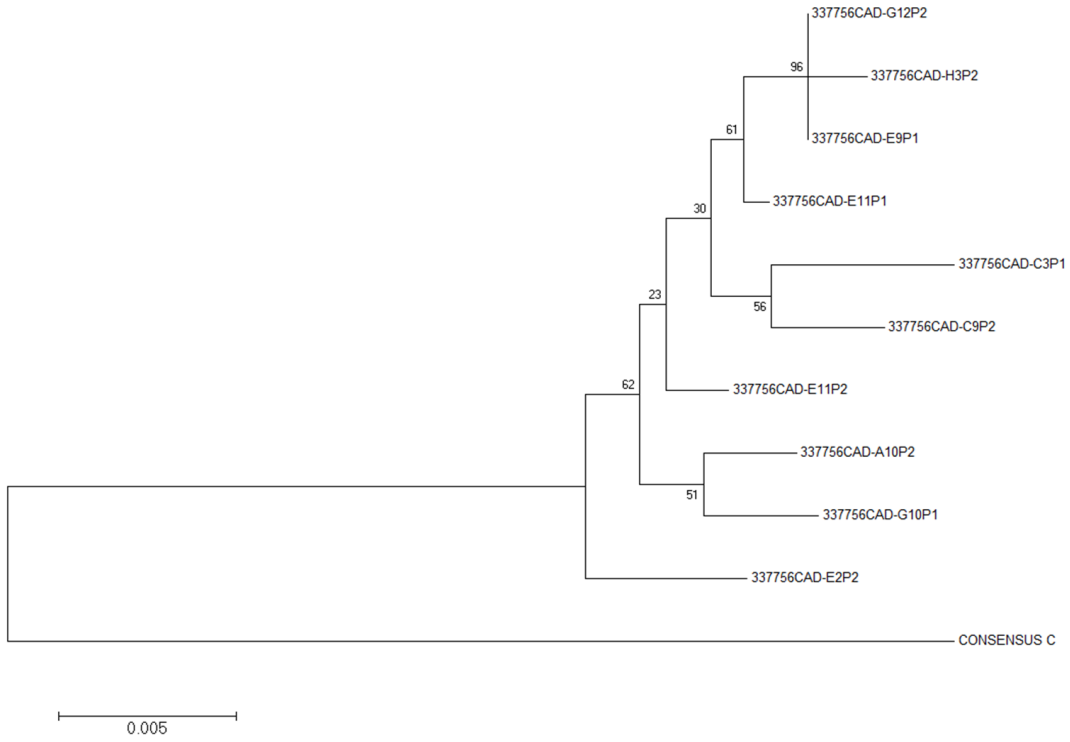


Figure 3.23: Neighbour-joining phylogenetic tree for patient 337756 constructed in Mega 5.0

Ten sequences were used to construct this phylogenetic tree, along with the root which was consensus C. Each branch demonstrates 10 different sequences from the same patient.

A log-linear growth curve was plotted in R software version 4.0.3 using the delta RT signal obtained, which was the RNA signal (RT reaction) with the DNA signal (NRT control) subtracted from it to measure cell-free HIV-1 virus from the VOA cell culture supernatants to determine whether replication had occurred or not. Post-CHER patient 337756 had three wells on the 6-well plate (STARLAB) that contained HIV-1-infected cells (Figure 2.15). Out of these three wells, well one and two both showed exponential growth according to Figure 3.24. Well one had detectable virus on day five and on every other day as well (day seven, 14, 21 and 28), while well two only showed detectable virus on day 14, 21 and 28 and at much lower concentrations. The third well from Post-CHER patient 337756 only had detectable virus on day 14 (Ct 39.49). Interestingly, in this well there was also DNA release from day 7 (Ct 39.63) from one of the two replicates in the RNA RT-qPCR assay of the NRT control.

Post-CHER patient 341622 had two wells on the 6-well plate (STARLAB) that contained HIV-1-infected cells (Figure 2.15). Virus was not recovered except for well two that had a single instance of low-level viral RNA release on day 14, which was not indicative of replication-competent virus recovery (Ct 39.73).

Samples from each well of the 6-well plates (STARLAB) from both Post-CHER patients at each time point sampled (day five, seven, 14, 21 and 28) were also assayed for the p24 antigen with the p24 ELISA (PerkinElmer®) to confirm that the wells that had exponential growth of HIV-1 at certain time points indeed contained replication-competent virus and to determine which assay detected replication-competent virus earlier. As can be seen in Figure 3.25, the substrate blank and negative controls included in the p24 ELISA kit (PerkinElmer®) had no colour change and was therefore negative. A positive control, included with the p24 ELISA kit (PerkinElmer®) was diluted from a 100 pg/mL to 50 pg/mL, to 25 pg/mL and to 12.5 pg/mL and each dilution was assayed in duplicate. All of the positive controls had a colour change to yellow, although the two lowest dilutions were very faint. The p24 ELISA (PerkinElmer®) has an analytical sensitivity of 3.5 pg/mL, although for this experiment specifically 12.5 pg/mL seemed like the lowest threshold that would be detected as the colour change was very faint and the OD reading was just above the cut off value according to the kit's specifications. No colour change was observed for any of the wells for patient 341622, which agrees with the RNA RT-qPCR assay results. For patient 337756, colour change was only observed on days 14, 21 and 28 from the well one samples.

Interestingly, there was no colour change observed for well two of patient 337756, even though this well showed exponential outgrowth of virus from day 14 to 28 with the RNA-RT-qPCR assay. This might be due to very low levels of RNA detected. Table 3.9 show how the p24 ELISA kit (PerkinElmer®) and the in-house RNA RT-qPCR assay differ in the detection of infectious virus. The in-house RNA RT-qPCR assay was able to detect virus on day five, whereas the p24 ELISA (PerkinElmer®) only detected virus on day 14 for the first time. This proved that the in-house RNA RT-qPCR assay was more sensitive than the p24 ELISA in detecting infectious virus earlier.

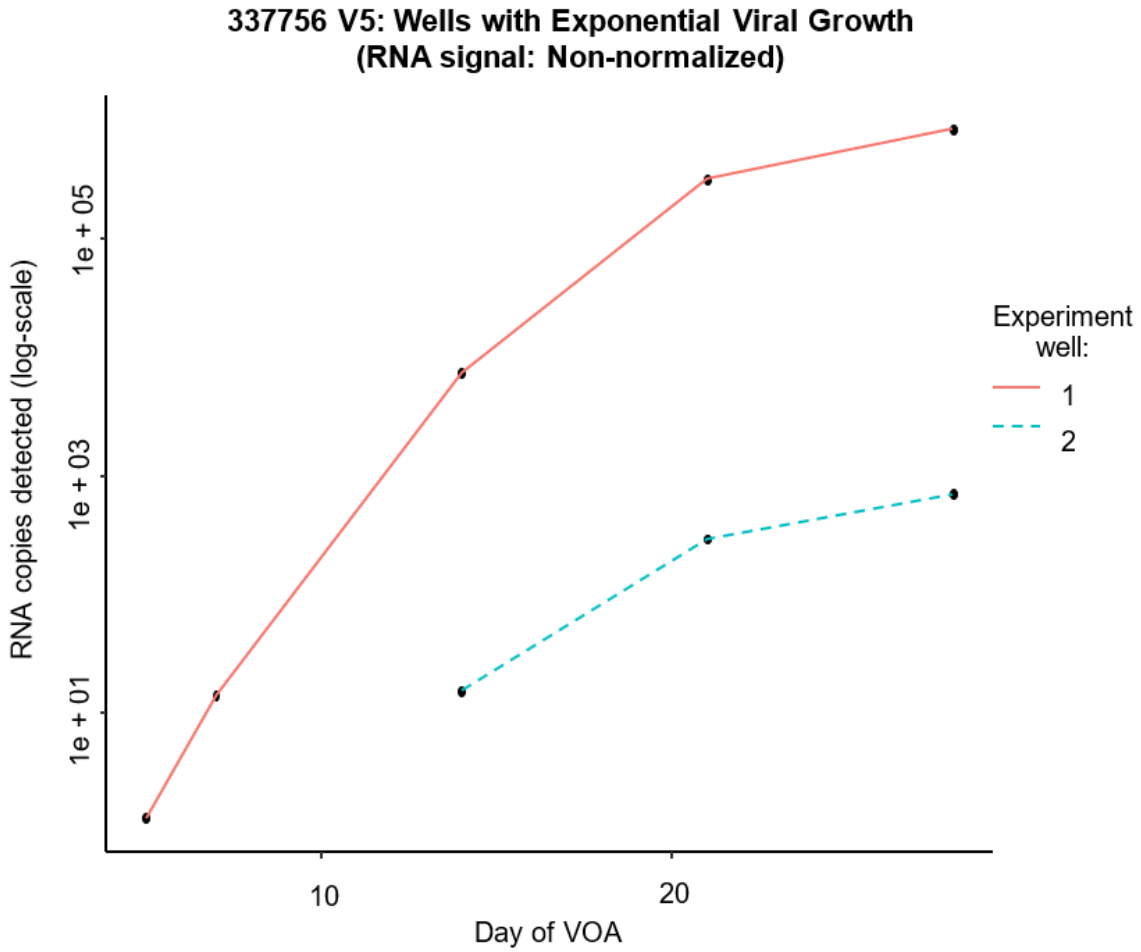


Figure 3.24: Log-linear growth curve for wells one and two from the 6-well plate (STARLAB) from Post-CHER patient 337756 on days five, seven, 14, 21 and 28

Table 3.9: Comparing results of the in-house RNA RT-qPCR assay to that of the p24 ELISA Kit (PerkinElmer®) for Patient 337756 (Well one and two) of the 28-day VOA

Day out of 28-day VOA	RNA Result with RNA RT-qPCR assay		p24 antigen result with p24 ELISA kit (PerkinElmer®)	
	Well 1	Well 2	Well 1	Well 2
5	+	-	-	-
7	+	-	-	-
14	++	+	+	-
21	+++	+	+++	-
28	+++	+	+++	-

"-" no signal for RNA and p24.
"+" RNA<1000 and for p24 a detected signal but less than the most concentrated positive control (an OD reading that falls between the OD reading obtained for the least concentrated positive control, 12.5 pg/mL, and the most concentrated positive control, 100 pg/mL).
"++" 1000<RNA<100 000 and for p24 a definitive positive but measurable (an OD reading greater than the most concentrated positive control, 100 pg/mL, but still within the limit of detection [reference range] for the specific microplate reader).
"+++" RNA>100 000 and for p24, an OD reading that is out of reference range.

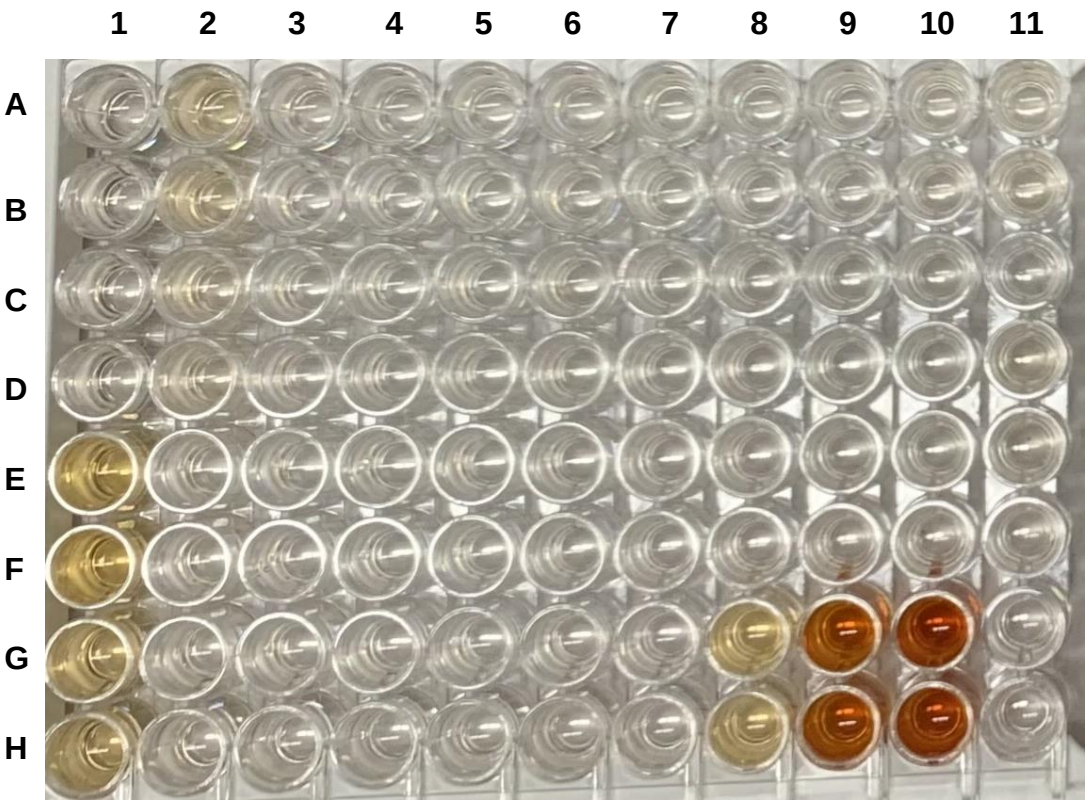


Figure 3.25: p24 ELISA (PerkinElmer®) results from patients 337756 and 341622 during the 28-day VOA

All of the wells for each patient was assayed (including the no NTC wells from both patients) and each patient well was assayed in duplicate. A1-substrate blank. B1, C1, D1-Negative control of the kit assayed in triplicate. E1, F1-Positive control at 100 pg/mL. G1, H1-Positive control at 50 pg/mL. A2, B2-Positive control at 25 pg/mL. C2, D2-Positive control at 12.5 pg/mL. Columns three, four, five and six up until row F included samples of patient 341622 over the 28 days. Columns six from row G up until column 11, row F included samples of patient 337756 over the 28-day VOA. G8 and H8-337756 well one, day 14. G9 and H9-337756 well one, day 21. G10 and H10-337756 well one, day 28.

Chapter 4

Discussion

4.1 Context and Summary of Findings

The aim of this study was to implement a more practicable VOA in a South African laboratory to be able to quantitate inducible and/or infectious virus from children undergoing suppressive cART. Infectious virus was recovered from two out of three VOA wells in one of the two currently suppressed, previously viraemic children, from the post-CHER cohort who had high concentrations of HIV-1 DNA. The in-house RT-qPCR assay was able to detect virus on day five for this child while the p24 ELISA, with an analytical sensitivity of 3.5 pg/mL, only had a positive result on day 14. Interestingly, the second VOA well for the same child had evidence of exponential outgrowth with the RT-qPCR assay, but the p24 had no positive signals. This proved that the RT-qPCR assay was more sensitive in detecting viral outgrowth compared to the p24 ELISA.

The latent reservoir in resting CD4+ T cells is known as the main barrier to curing HIV-1 infection. While many PCR based assays are utilised to measure the latent reservoir most of them have the major disadvantage of detecting defective proviruses that do not present a barrier to cure. Examples of such assays are: qPCR assays for HIV-1 DNA (Rouzioux et al., 2014). These assays are some of the easiest, but they detect total proviral DNA which includes defective proviruses (Ho et al., 2013). Another example of a PCR based assay is the Alu-PCR that measures integrated HIV-1 DNA, although this assay also detects defective proviruses and once again overestimates the size of the latent reservoir (Brady et al., 2013). An assay that is able to distinguish between likely intact and defective proviruses is the intact proviral DNA assay (IPDA), however this assay only gives an approximation of the likely intact reservoir as it is not able to detect all defective viruses (Bruner et al., 2019). Then there are also assays that measure residual viraemia, which is low level viraemia below the threshold of commercial assays, such as HIV-1 plasma RNA single copy assays (SCA) (Palmer et al., 2003). However only a small fraction of reservoir cells are activated and would produce virions at a particular instance, which makes this assay insensitive. It is also non-specific as there is new evidence that some non-intact proviruses could be

transcribed to RNA, which could be packaged into virions (as long as gag-p24 is produced) and released from host cells. These non-intact proviruses would not be able to complete viral replication (Imamichi et al., 2020).

The TILDA, that measures cell-associated *tat*/*rev* ms-RNA, is another assay that has been used to measure the size of the latent reservoir. In this assay, total CD4+ T cells are maximally activated to induce HIV-1 DNA transcription. This assay is valuable in that it is less time-consuming compared to the standard qVOA, it only requires small blood volumes and it does not require outgrowth of virus which makes it well suited for longitudinal studies. However, as not all induced proviruses are replication-competent, it overestimates the reservoir relative to assays that measure replication-competent reservoirs (Procopio et al., 2015).

Therefore, the qVOA still remains the gold standard to quantify replication-competent virus, produced by latently infected cells as due to the high threshold of the traditional 24 antigen assays, it requires exponential growth before the p24 antigen signal would become positive. Although, the qVOA still has multiple disadvantages, such as being costly (due to measuring outgrowth with p24 ELISA kits), time-consuming (as it requires culture time of two to three weeks or sometimes even longer for viral outgrowth) and labour-intensive (with proper planning before starting the assay and having to maintain the culture during the assay). The qVOA also requires large blood volumes from HIV-1-infected patients to plate the cells at serial dilutions, it underestimates the size of the latent reservoir (since not all intact-non-induced proviruses are detected after one round of stimulation) and the assay is variable due to donor variability in cells for HIV-1 expansion (Siliciano & Siliciano, 2005) (Bruner et al., 2015) (Massanella & Richman, 2016).

There have been adaptations of the standard qVOA to attempt to optimise the assay. Laird et al. (2013) have developed a rapid VOA that was able to quantify the frequency of cells that could release replication-competent virus after cellular activation. The VOA implemented by Laird et al. (2013) utilised MOLT-4 CCR5+ cells for virus expansion that generated results that were statistically comparable by eliminating the variability that comes with using CD8+ depleted lymphoblasts from different donors. This assay also utilised a quantitative RT-PCR specific for polyadenylated HIV-1 RNA for virus detection. This RT-

PCR proved to be more sensitive and cost-effective to detect HIV-1 replication compared to the expensive ELISA methods.

Hosmane et al. (2017) implemented a multiple stimulation VOA (MS-VOA) and demonstrated that latently infected cells could proliferate in response to mitogens without producing virus; therefore, generating progeny cells that could release infectious virus. Resting CD4+ T cells from patients on long-term ART were stimulated more than once to detect virus released from cells that had proliferated in response to a previous stimulation without producing virus. They found that additional isolates of replication-competent HIV-1 could be obtained after a second, third and fourth additional rounds of T cell activation. Similar to Laird et al. (2013), they also utilised MOLT-4 CCR5+ cells as target cells for virus expansion. Their results agree with what Ho et al. (2013) found in that one round of stimulation underestimates the size of the latent reservoir as not all intact-non-induced proviruses are released.

Zhang et al. (2020) designed a simplified *all-trans* retinoic acid (ATRA)-based qVOA. *All-trans* retinoic acid (ATRA) is a key modulator of immunity via the modulation of T-cell effector functions, it also has the ability to enhance cell-to-cell transmission in culture to activate HIV-1. The use of ATRA in the ATRA-qVOA, points to the potential of other molecular boosters to enhance HIV-1 outgrowth to optimise the current qVOAs. Here, memory CD4+ T cells were isolated from ART suppressed patients (as these cells have been recognized to be enriched in HIV-1 reservoirs), the cell culture conditions were optimised (by splitting the cultures every three days) and the cultures were supplemented with ATRA for 12 days. They found that by splitting the memory CD4+ T cells every three days optimal density in cell culture was maintained, and by supplementing the cell cultures with ATRA significantly improved the efficiency of viral outgrowth. Therefore, this simplified ATRA-based qVOA shows value in that cell culture time was reduced compared to the standard qVOA and no feeder cells or indicator cell lines were needed.

Stone et al. (2020) compared six different “next-generation” VOAs by PCR or p24 to assess their suitability as scalable substitutions for the standard qVOA. This was done by comparing next-generation qVOAs to the standard qVOA. Extra-Poisson variation at laboratory, aliquot and batch levels were estimated using Markov chain Monte Carlo methods. Their data demonstrated that next-generation qVOAs could be suitable substitutions for more laborious

qVOAs, as it provides better sensitivities and dynamic ranges and it also provides a high throughput which is beneficial in studying eradication strategies.

Stuelke et al. (2020) developed the Digital ELISA Viral Outgrowth (DEVO) assay. This assay utilises an ultrasensitive p24 readout method that is capable of detecting femtogram concentrations of HIV-1 p24, compared to the traditional ELISAs that can only detect p24 at picogram levels. Each DEVO assay requires 8-12 x 10^6 resting CD4+ T cells from patients to be plated at limiting dilutions. The standard qVOA procedure is followed (except either MOLT-4 CCR5+ cells or CD8+ depleted lymphoblasts can be used for HIV-1 expansion) and the virus is cultured for eight to 12 days. On day eight, HIV-1 p24 is measured by the single molecule array, ultrasensitive p24 assay (HIV p24 Simoa®, Quanterix) and IUPM are calculated with the maximum likelihood method. They found that all the DEVO assays performed were able to detect HIV-1 p24 eight days after stimulation. Also, the IUPM value on day eight was similar or even higher than those obtained with the standard qVOA on day 15. This study also showed that IUPM values obtained with the DEVO assay were similar, with or without the addition of HIV-1 expansion cells, or CD8+depleted donor lymphoblasts. This assay is more time efficient compared to the standard qVOA and it does not require HIV-1 target cells to expand the virus in culture.

Patient 337756 had three wells in a 6-well plate that was assayed for replication-competent virus. Well one and two both had exponential growth according to the in-house RNA RT-qPCR assay. Well one already had HIV-1 RNA detection on day five while well two first had detectable HIV-1 RNA on day 14. The fact that patient 337756 had a high HIV-1 DNA diversity as well as more than one episode of detectable viraemia during suppressive cART might have contributed to outgrowth of virus. As comparison with the RNA RT-qPCR assay the wells were also assayed for p24 with the p24 ELISA Kit from PerkinElmer®. The p24 ELISA kit from PerkinElmer® has an analytical sensitivity of 3.5 pg/mL; however, when the samples were assayed for p24 during the 28-day VOA, the lowest threshold of detection seemed to be 12.5 pg/mL as the OD obtained was just above cut off value according to the kit's specifications. To achieve a positive p24 signal, high concentrations of RNA were required. This can be confirmed by the fact that the first positive p24 signal was around 10 000 copies of RNA from well one of patient 337756 on day 14. The use of a RT-qPCR assay to detect viral outgrowth proved to be more sensitive compared to the p24 ELISA kits,

as it detected outgrowth earlier, on day five, whereas the p24 ELISA first detected outgrowth on day 14, this was similar to what Laird et al. (2013) found.

Also, outgrowth, as evident from exponential growth, was detected for well two for patient 337756 with the in-house RNA RT-qPCR assay, despite this being below the threshold of the p24 signal as obtained with the p24 ELISA kit (PerkinElmer®). This could represent virus that had low fitness and a slower replication rate than what was needed to expand the virus to beyond the limit of detection of the p24 ELISA (PerkinElmer®). This emphasises the lack of sensitivity in traditional p24 assays and the importance of designing p24 assays with greater sensitivities, such as what Stuelke et al. (2020) did. By using a more sensitive p24 assay, such as the Simoa, virus with a slower replication rate could be detected easier and earlier, but on the contrary it may be prone to false positives also (either analytical error or due to release of non-replication-competent virus). It is important to note that the p24 ELISA and the in-house RNA RT-qPCR assay have different targets. The in-house RNA RT-qPCR assay targets cell-free HIV-1 RNA in cell culture supernatant (specifically the *integrase* region in the HIV-1 genome). Targeting cell-free RNA is advantageous as it reflects the capacity to induce translation and form and release virions (Massanella & Richman, 2016). It might be possible that this RNA RT-qPCR assay could detect small amounts of replication-defective virus that are released after stimulation, which could result in a false positive readout. However, if the positive cases had lower Ct values, were positive for HIV-1 RNA on successive days and showed clear exponential growth it would point to a likely true positive for viral outgrowth. The p24 antigen is a major structural core component of HIV-1 and it is present in high levels when cells were recently infected which demonstrates the presence of infectious virus. The p24 ELISA is seen as the gold standard to prove replication-competence of HIV-1. However, having an RNA RT-qPCR assay that is much more cost-effective compared to p24 ELISA kits and that is able to detect viral outgrowth earlier could be a very valuable tool in eradication strategies.

Also, similar to what Laird et al. (2013) did, the VOA in this study utilised the MOLT-4 CCR5+ cell line to expand the virus instead of CD8+ depleted lymphoblasts. The use of this cell line made the assay less labour-intensive, more reproducible, and comparable, compared to the gold standard qVOA.. However, by using qVOAs such as the ATRA-based qVOA (Zhang et al., 2020) that does not require cell lines for virus propagation or the DEVO assay that also showed potential to expand virus without any target cells (Stuelke et al., 2020), the VOA

could be made even less labour-intensive (as cell lines require two weeks of culture time before starting the VOA).

Another difference of the VOA employed in this study compared to the gold standard qVOA is that here HIV-1-infected total CD4+ T cells were isolated from patients and the cells were only tested at a single concentration (1 x 10^6 cells/well). Whereas with the gold standard qVOA, HIV-1-infected patient cells are tested in serial dilutions to determine the frequency of latently infected cells that harbour replication-competent virus. In this case the VOA from this study could be valuable in working with children where small blood volumes are obtained and most other adapted/optimised qVOAs as described earlier still require relatively high concentrations of either resting CD4+T cells (Laird et al., 2013) (Hosmane et al., 2017) (Stuelke et al., 2020) or memory CD4+ T cells (Zhang et al., 2020). Activating CD4+ T cells in bulk, could be useful for a qualitative assay to test various concentrations of LRAs (Massanella & Richman, 2016). Also, by evaluating replicates of large numbers of CD4+ T cells, patients with very low levels of cell-associated HIV-1 DNA or RNA could be studied for replication-competent virus. In the case of small sample volumes, using an assay like the TILDA (Procopio et al., 2015) could be valuable as this assay only requires 10 mL of blood, however this assay measures inducible virus and therefore replication-competence of reactivated proviruses cannot be assessed fully.

Interestingly, the third VOA well of patient 337756 had detectable HIV-1 RNA on day 14, at a Ct of 39.49, which could have been indicative of low-level inducible virus. As most HIV-1 latently infected cells do not transcribe viral RNA, the presence of ms-RNA reflects the ability of latently infected cells to produce virus (inducible virus), but inducible virus does not imply replication_competence because it only accounts for transcriptionally competent proviruses (Fischer et al., 2004) (Massanella & Richman, 2016). The same well also had HIV-1 DNA detected at low levels (Ct 39.63) on day seven. As the cell culture supernatant was centrifuged before assessing for viral outgrowth, a possible explanation for DNA detection in the supernatant could be due to the HIV-1 infected CD4+ T cells from the patient dying of syncytium formation which caused the cells to lyse releasing HIV-1 DNA into the cell culture supernatant. There have been early reports that demonstrated that HIV-1 Env can engage receptors on neighbouring cells to trigger cell to cell fusion events that gives rise to giant, multinucleated cells (Lifson et al., 1986) (Kowalski et al., 1987).

For patient 341622, no infectious virus was recovered and a weak HIV-1 RNA signal was detected from well two on day 14 which could have also been low level release of inducible virus as explained earlier. Also, since only one round of T cell stimulation was performed, it is possible that not all intact proviruses were stimulated to be released, referred to as intact-non-induced proviruses. Therefore, the VOA from this study also underestimated the size of the latent reservoir. By adapting the assay to perform multiple stimulations like the MS-qVOA (Hosmane et al., 2017), a more accurate reservoir could be measured. However, the MS-qVOA could be more protracted in that cells have to be stimulated after eight days and after each successive stimulation, the cells have to still be cultured for 21 days which leads to a long cycle of cell culture and since most non-induced proviruses are known to be defective, this VOA does still provide an accurate picture of the true latent reservoir.

In this study a large number of total CD4+ T cells were assayed in replicates of two or three at most and despite the high concentration of CD4+ T cells in each well, only one well out of three of the one child yielded high enough concentrations of virus to be detected by p24, while the other well was positive for RNA with the inhouse RNA RT-qPCR assay but not for p24 and the second patient had no outgrowth detected with p24 or the RNA RT-qPCR assay out of two wells. This demonstrates the difficulty of measuring true latent reservoir in children who initiated therapy early as this reservoir seems to be small and extremely difficult to recover.

4.2 Strengths of this Study

The key strengths of this study include that a simple VOA was implemented that was able to show that infectious HIV-1 could be recovered from a child undergoing suppressive ART. This study also utilised a simple, yet sensitive RNA RT-qPCR assay to measure viral outgrowth. This assay was much more affordable to implement compared to using expensive p24 ELISA kits. The RNA RT-qPCR assay was also able to detect viral outgrowth earlier compared to the HIV-1 P24 ELISA kit. This study also utilised a continuously growing MOLT-4 CCR5+ cell line instead of CD8+ depleted donor lymphoblasts to expand HIV-1 in culture. The use of this cell line makes the VOA more reproducible and less variable between different laboratories, it also makes the VOA less labour intensive as there was no need for additional CD8+ depleted lymphoblasts from uninfected donors to use as target cells for the VOA. Also, the MOLT-4 CCR5+ cell line is only added on day two of the VOA while CD8+

depleted lymphoblasts need to be added twice (on days two and seven). Also, when resting CD4+ T cells are used, productively infected cells are excluded; therefore CD69+, CD25+ and HLA-DR+CD4+ T cells are removed from cell culture. These markers, however, do not only identify the truly activated CD4+ T cells, but also proliferating cells (HLA-DR+) and regulatory T cells (CD25+) which could also add relevant contribution to HIV-1 persistence (Massanella & Richman, 2016). In this case, using total CD4+ T cells from HIV-1-infected patients might be valuable.

4.3 Limitations of this Study and Challenges Faced

Even though the VOA used in this study was simplified in multiple ways, there were still limitations that were similar to those of the gold standard qVOA.It was still labour intensive and time consuming in that much planning had to be done before starting the assay. Target cells for HIV-1 propagation needed to be at adequate concentrations, which meant that MOLT-4 CCR5+ cells had to be grown in culture two weeks before the time. Donors for feeder cells had to be obtained and then logistics had to be sorted out regarding irradiation of these cells as there was no irradiator in the laboratory. This VOA also required relatively large amounts of PBMCs to be able to isolate sufficient total CD4+ T cells for plating (generally only 15%-24% of CD4+ T cells were recovered out of total PBMCs). Also, just like the qVOA, this simple VOA might underestimate the true size of the latent reservoir since only one round of stimulation with PHA was performed and one round of stimulation is not enough to activate all intact proviruses (Bruner et al., 2015). The quantity of latently infected cells could not be determined in this study since HIV-1-infected patient cells were only tested at a single concentration (1×10^6 cells/well). Another limitation of this study is that only two samples were assayed for replication-competent virus and therefore, patients with a range of low to higher levels of total cell-associated DNA could not be investigated.

Some challenges were that, when working with child samples, one is limited to the amount of blood that is allowed to be drawn, since up to 10% of the total blood volume is allowed to be drawn over eight weeks (Howie, 2011). This made it difficult to isolate enough CD4+ T cells to assay as many wells at a million cells per well as possible (at most three wells were assayed). There were also challenges in obtaining all the reagents and equipment necessary to implement this assay. It was also challenging to implement this assay in a laboratory where it had not been implemented before and having a limited number of people

with experience in this specific assay to assist in training and providing advice. There were also delays in waiting for reagents to arrive, equipment that was faulty and the SARS-CoV-2 epidemic prevented access to the laboratory.

4.4 Future Perspective

It might be valuable to test more ART suppressed children, over a range of proviral loads, with the VOA for replication-competent virus to strengthen the results of this study. A lot can be learned from new qVOAs that have recently been designed, as explained earlier. For future studies, this VOA could be optimised even more by performing multiple stimulations to be more accurate in measuring the true latent reservoir, such as the MS-qVOA. However to shorten the time of the MS-qVOA a more sensitive detection method could be used such as the RT-qPCR developed by Laird et al. (2013) or the in-house RT-qPCR assay from this study. A sensitive p24 assay could also be designed such as the Simoa that would be able to detect infectious virus as early as eight days after stimulation. Future studies could also look into avoiding the use of expansion cells or cell lines like what was done in the ATRA-based qVOA (Zhang et al., 2020) and the DEVO assay (Stuelke et al., 2020).

Nevertheless, this VOA was able to detect replication-competent virus as it measured cell-free RNA with a sensitive RT-qPCR assay. As mentioned before cell-free RNA is a more specific marker in detecting replication-competent virus as it reflects the ability to induce translation and therefore to form and release infectious virions, whereas cell-associated RNA is a more sensitive marker to assess inducible virus recovery but not all inducible viruses are replication-competent. This VOA that utilises MOLT-4 CCR5+ cells and a sensitive RNA RT-qPCR assay could add great value in pharmacological studies to reverse latency to eliminate infected cells.

Chapter 5

Conclusion

In conclusion, in line with this study's aim and objectives, a simple virus recovery assay was implemented in a South African laboratory setting that was able to recover infectious virus from a child undergoing suppressive cART. This VOA utilised an RNA RT-qPCR assay that was able to detect exponential outgrowth earlier as compared to the p24 ELISA. The VOA implemented in this study also utilised a continuously growing T lymphoblast cell line, MOLT-4 CCR5+, that expressed the CD4+ receptor as well as both CCR5+ and CXCR4+ co-receptors to expand HIV-1 in culture instead of donor CD8+ depleted lymphoblasts. This made the assay much more practicable, more cost-effective and more reproducible. Since only two children were assayed for infectious virus, it might be valuable to assay more children over a range of proviral loads. Even though this VOA proved to be more reproducible and practicable than the gold standard qVOA, it still underestimates the true size of the latent reservoir and could do with some more optimizing as it is still a labour-intensive assay. This study highlights the importance of developing simpler qVOAs to measure the latent reservoir in order to evaluate eradication strategies in clinical trials. This simplified VOA might be a valuable tool in evaluating the success of strategies to perturb or eradicate the HIV-1 latent reservoir in children on suppressive cART.

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Addenda A

Addendum A1 Amplification procedure from pMJ4

Primers targeting the p31 region of the *pol* gene in the linearised MJ4 plasmid:

Primer Name	Sequence (5'-3')	Position (bp)*
Forward Primer	CCCTACAATCCCCAAAGTCA	4653-4672
Reverse Primer	CACAATCATCACCTGCCATC	5070-5051

*Location based on HXB2 HIV-1 reference genome, GenBank accession number: K03455.

Calculations for the end-point PCR to generate a 418 bp amplicon, containing an HIV-1 subtype C *integrase* insert, from the linearised MJ4 plasmid:

Reagent	Final concentration	Volume per reaction (µL)
Nuclease-free water[a]	-	20.25
GoTaq flexi buffer[b]	1X	10
dNTPs[c]	0.20 mM each	1
$MgCl_2$[d]	2 mM	4
GoTaq G2 Hot Polymerase[e]	3.75 U	0.75
Forward Primer	0.40 µM	2
Reverse Primer	0.40 µM	2
DNA	20 ng	10
Total	-	50

[a]Qiagen. [b]Promega Corp., Madison, USA. [c]Thermo Fisher Scientific, Waltham, USA. [d]Promega Corp., Madison, USA. [e]Promega Corp., Madison, USA.

Cycle parameters for end-point PCR to generate a 418 bp amplicon, from the linearised MJ4 plasmid:

Step	Temperature	Time	
Initial Denaturation	94°C	2 minutes	
Denaturation	94°C	1 minute	30 cycles
Annealing	44°C	1 minute	
Extension	72°C	30 seconds	
Final Extension	72°C	20 minutes	
Hold	4°C	∞	

Addendum A2 Recipe to make up 1X SB Buffer

1. Make up 20X SB Buffer first = 8 g Sodium Hydroxide (Merck) + 47 g Boric Acid (Merck) dissolved in 1 L Milli-Q water (Merck).
2. Then from 20X SB Buffer make 1X SB Buffer = Add 50 mL 20X SB Buffer to 950 mL Milli-Q water (Merck).

Addendum A3 Incubation conditions for the ligation step of the cloning procedure

Step	Temperature	Time
Stage 1[a]	25°C	1 hour
Stage 2[b]	4°C	1 hour (8 cycles)
Stage 3[c]	75°C	5 minutes
Hold	4°C	∞

[a]Incubate at room temperature. [b]Incubate for 8 hours to obtain maximum transformants. [c]Deactivation of T4 DNA Ligase enzyme (Thermo Fisher Scientific, Waltham, USA).

Addendum A4 Recipes for LB agar and LB broth solution

Recipe to make LB agar medium petri dishes:

1. To 10 g LB Broth (Sigma-Aldrich) and 7.5 g LB agar (Sigma-Aldrich) add Milli-Q water (Merck) up to 500 mL.
2. Autoclave for 30 minutes and leave to cool down completely.

3. When cooled down completely, add ampicillin (Tocris Bioscience) to the LB agar medium:

   C1V1 = C2V2

   (100 mg/mL)(V1) = (0.05 mg/mL)(500 mL)

   V1 = 250 µL Ampicillin (Tocris Bioscience) to add to the 500 mL LB agar medium
4. Pour the LB agar medium plates and let it solidify.
5. Store the LB agar medium plates upside down at 4°C until used.

Recipe to make LB broth solution:

1. To 10 g LB broth (Sigma-Aldrich) add Milli-Q water (Merck) up to 500 mL.
2. Autoclave for 30 minutes and leave to cool down completely.
3. When cooled down completely, add ampicillin (Tocris Bioscience) to the LB broth solution:

   C1V1 = C2V2

   (100 mg/mL)(V1) = (0.05 mg/mL)(500 mL)

   V1 = 250 µL Ampicillin (Tocris Bioscience) to add to the 500 mL LB broth solution
4. Store the LB broth solution at 4°C until used.

Addendum A5 Sequencing of plasmid

M13/pUC Sequencing Primers:

Primer Name	Sequence (5'-3')	Position (bp)*
M13/pUC forward sequencing primer (-20), 17-mer[a]	GTAAAACGACGGCCAGT	599-614
M13/pUC reverse sequencing primer (-26), 17-mer[b]	CAGGAAACAGCTATGAC	735-751

[a]Catalog number: SO100. [b]Catalog number: SO101. *Position based on the MCS of the pTZ57R/T cloning vector from the InsTAclone™ PCR Cloning kit (Thermo Fisher Scientific).

Calculations for the sequencing PCR reaction:

Reagent	Final concentration	Volume per reaction (µL)
Nuclease-free water[a]	-	4
Reaction Mix		1
EDTA buffer[b]	1X	3
M13/pUC sequencing primers	5 pmol/µL	1
Ligated DNA sample (Pure plasmid DNA)	15-25 ng/µL	1
Total		10

[a]Qiagen. [b]Thermo Fisher Scientific, Waltham, USA.

Cycle parameters for sequencing PCR reaction:

Step	Temperature	Time	
Denaturation	96°C	0.10 seconds	25 cycles
Annealing	50°C	0.05 seconds	
Extension	60°C	4 minutes	
Hold	4°C	∞	

Addendum A6 Linearisation of plasmid DNA

Calculations for the linearisation reaction:

Reagent	Final Concentration	Volume per reaction (µL)
Nuclease-free water[a]	-	16.30[e]
Buffer H (10X Buffer)[b]	1X	2
Acetylated Bovine Serum Albumin[c]	10 µg/µL	0.20
DNA After adding DNA, mix by pipetting	0.20-1.50 µg	1[f]
EcoRI[d]	10 u/µL	0.50
Total		20

[a]Qiagen. [b]Promega Corp., Madison, USA. [c]Promega Corp., Madison, USA. [d]Promega Corp. [e]This volume changes according to the volume of DNA in the reaction. [f]This volume changes depending on the starting concentration of the DNA based on the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific) and according to the amount of DNA being put into this reaction (anything between 0.2-1.5 µg of DNA).

Cycle parameters for the linearisation reaction:

Step	Temperature	Time
Incubation of plasmid at restriction enzyme's optimum temperature	37°C	2 hours
Inactivation of restriction enzyme	85°C	5 minutes

Addendum A7 TDR Buffer Recipe

Combine 5 mM Tris-HCl (Sigma-Aldrich), 100 mM DTT (Thermo Fisher Scientific) and 10 000 units/mL recombinant RNasin ribonuclease inhibitor (Promega Corp.) to make this buffer.

Addendum A8 RNA RT-qPCR Assay

RT and NRT master mixes for cDNA synthesis:

Reagent	**Final concentration**	**RT control cocktail (cDNA synthesis):** Volume per reaction (µL)	**NRT control cocktail (DNA signal evaluation):** Volume per reaction (µL)
Nuclease-free water[a]	-	8.10	8.20
$MgCl_2$[b]	5 mM	6.00	6.00
dNTPs[c]	0.50 mM	0.60	0.60
DTT[d]	1 mM	0.20	0.20
Random Hexamers[e]	0.15 µg/reaction	1.50	1.50
Taqman buffer A (In-house)	1X	3.00	3.00
Recombinant RNasin[f]	20 U	0.50	0.50
Stratagene RT[g]	20 U	0.10	No RT added
Total master mix		20	20
Sample RNA		10	10
Total for reaction		30	30

[a]Qiagen. [b]Promega Corp. [c]Bioline, London, UK. [d]Thermo Fisher Scientific. [e]Promega Corp, Madison, USA (Make sure 160 µL nuclease-free water [Qiagen] was added before first use). fPromega Corp. gAgilent, Santa Clara, USA.

Taqman Buffer A Recipe:

Combine 1 M Tris-HCl (Sigma-Aldrich) + 1 M potassium chloride (KCl) (Sigma-Aldrich, St. Louis, USA) + 100% Tween® 20 (Sigma-Aldrich, St. Louis, USA) + Nuclease-free water (Qiagen) and store in 1 mL aliquots at -20°C.

Cycle conditions for cDNA synthesis for the RNA RT-qPCR assay:

Step	Temperature	Time
1	25°C	15 minutes
2	42°C	40 minutes
3	85°C	10 minutes
4	25°C	30 minutes
5	4°C	∞

RNA RT-qPCR assay real-time primers & probe:

Primer Name	Sequence (5'-3')
Integrase forward primer	TTTGGAAAGGACCAGCCA
Integrase reverse primer	CCTGCCATCTGTTTTCCA
Integrase probe	/56-FAM/AAAGGTGAA/ZEN/GGGGCAGTAGTAATACA/3IABkFQ/

qPCR master mix for RNA RT-qPCR assay:

Reagent	Final concentration	Volume per reaction (µL)
LightCycler® 480 Probes Master Mix*	1X	19.50
RT qPCR Forward *Integrase* Primer	400 nM	0.20
RT qPCR Reverse *Integrase* Primer	400 nM	0.20
RT qPCR *Integrase* Probe	200 nM	0.10
Total Cocktail		20.00
Sample cDNA/RT mix		30.00
Final Total Volume in each well		50.00

*Roche Holding AG, Basel, Switzerland.

Cycle conditions for the qPCR reaction of the RNA RT-qPCR assay:

Step	Temperature	Time	
1	95°C	10 minutes	
2	95°C	15 seconds	45 cycles
3	60°C	1 minute	
4	4°C	∞	

Addenda B

Addendum B1 Cell culture media

Wash media recipe (Also staining buffer recipe):

Add 2% HI & GI FBS (Thermo Fisher Scientific) to 1X DPBS (Thermo Fisher Scientific).

Cryopreservation media:

10% DMSO (Sigma-Aldrich) and 90% HI & GI FBS (Thermo Fisher Scientific).

Addendum B2 Preparation of T cell growth factor

T cell growth factor cell culture media:

Add 2.5% HI Human Serum AB male HIV tested (Biowest) and 1X Penicillin-Streptomycin (Biosciences) to RPMI 1640 medium with L-Glutamine and phenol red (Lonza Group Ltd).

Cell Culture Media for Human PBMCs:

To RPMI 1640 medium with L-Glutamine and phenol red (Lonza Group Ltd), add 20% HI & GI FBS (Thermo Fisher Scientific) and 100 U/mL Gibco™ Recombinant IL-2 (Thermo Fisher Scientific).

Addendum B3 VOA media

MOLT-4 CCR5+ cell culture recovery media:

To RPMI 1640 medium without L-Glutamine and phenol red (Lonza Group Ltd) add 20% HI & GI FBS (Thermo Fisher Scientific) and 1X GlutaMAX™ (Biosciences).

MOLT-4 CCR5+ media:

To RPMI 1640 medium without L-Glutamine and phenol red (Lonza Group Ltd) add 20% HI & GI FBS (Thermo Fisher Scientific), 1X GlutaMAX™ (Biosciences) and 1X Penicillin-Streptomycin (Biosciences).

STCM Recipe:

To RPMI 1640 medium without L-Glutamine and phenol red (Lonza Group Ltd) add 20% HI & GI FBS (Thermo Fisher Scientific), 1X GlutaMAX™ (Biosciences), 1X Penicillin-Streptomycin (Biosciences), 100 U/mL Gibco™ Recombinant IL-2 (Thermo Fisher Scientific) and 2% TCGF (In-house).

Addendum B4 Formulas

Separation Index Formula:

$$Seperation\ Index = \frac{(MFI_1 - MFI_2)}{(84\%\ MFI_2 - MFI_2)/0.995}$$

MFI_1= median fluorescent intensity of positive population

MFI_2= median fluorescent intensity of negative population

Stain Index Formula:

$$Stain\ Index = \frac{(MFI_1 - MFI_2)}{2\ x\ SD\ negative}$$

MFI_1= median fluorescent intensity of positive population

MFI_2= median fluorescent intensity of negative population

SD negative= standard deviation of the negative population