BOTSWANA INTERNATIONAL UNIVERSITY OF SCIENCE & TECHNOLOGY

School of Life Sciences

IMMORTALIZATION OF PRIMARY LAMB KIDNEY CELLS TOWARDS A CONTINUOUS CELL LINE FOR PROPAGATION OF FOOT AND MOUTH DISEASE VIRUS

by

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BSc (Biology) (UB),

A Dissertation/Thesis Submitted to the Faculty of Sciences in Partial Fulfilment of the Requirements for the Award of the Degree of Master of Science in Biological science and Biotechnology of BIUST

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July, 2025

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List of abbreviations

BHK- Baby hamster kidney cells

BTY - Bovine thyroid cells

BVI- Botswana Vaccine Institute

CPE - Cytopathic effect

CRISPR - Clustered regularly interspaced short palindromic repeats

Ct - Cycle threshold

DMSO - Dimethyl sulfoxide

FMD - Foot and mouth disease

FMDV - Foot and mouth disease virus

hTERT - Human telomerase reverse transcriptase

MEM - Minimal essential media

RM - Primary lamb kidney

RNA - Ribonucleic acid

RT- qPCR - Reverse transcription quantitative polymerase chain reaction

SAT - Southern African Territories

RLFMD - Reference Laboratory for Foot and Mouth Disease

SV40LT - Simian virus 40 large T antigen

TERT - Telomerase reverse transcriptase

$TCID_{50}$- Tissue culture infectious dose at 50%

WOAH- World Organization for Animal Health

VNT – Virus neutralization test

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Acknowledgements

I would like to extend my gratitude to the **Government of Botswana** and the **Botswana Vaccine Institute (BVI)** for the financial support and the time that they availed me to pursue this project. My utmost gratitude goes to the **Botswana International University of Science and Technology** for giving me the opportunity (non-financial support) to pursue MSc in Biological Sciences and Biotechnology. Thirdly, I would like to extend my appreciation to my Supervisors, **Dr Kebaneilwe Lebani**, **Dr Elliot Mpolokang Fana** and **Dr Kabo Masisi** for having been key players in guiding me through this journey to ensure quality output of this research. Immense gratitude is expressed to **Dr Joseph Hyera, Tefo Mogotsi, the BVI quality control team, Aobakwe Rapharing** and **Petronella Mosholombe** for their input and support through the experiments. I would also like to appreciate Mr **Mbatshi Hunyepa** from the BVI procurement team, for his patience, support, and promptness towards sourcing all the reagents that I required for my project. I would also love to acknowledge the Department of Biological Sciences and Biotechnology and my only classmate (MSc class of 2023-2025) **Umuna Maendo** for the support that she showed me during my studies. I would also like to thank **my family** and **my brother Nonofo Thapelo** for their unparalleled support and motivation during my studies. Finally, I would like to thank the **Almighty God** for all the answered prayers.

Abstract

Foot and mouth disease (FMD) is a highly contagious disease of great economic importance. The disease is caused by foot and mouth disease virus (FMDV). Diagnosis of FMD is done through tests such as virus isolation (VI), enzyme linked immunosorbent assay (ELISA), virus neutralisation tests (VNT) and polymerase chain reaction (PCR) platforms. Tests such as VI and VNT require the use of cells that are susceptible to infection with FMDV. Cells that can be used for FMDV culture include primary cells and cell lines developed for diagnostic applications. Cells such as primary lamb kidney cells have been shown to be very sensitive to infection with FMDV, but the cells are short lived, laborious to prepare, and give variable results due to the use of different batches. Notwithstanding, the sensitivity of primary lamb kidney cells to infection by FMDV can be taken advantage of and developed into a cell line. Primary lamb kidneys cells' lifespan can be increased through exogenous introduction of telomerase reverse transcriptase (*TERT*). In this study, transduction was conducted on primary lamb kidney cells at 80% confluency using recombinant lentivirus to over express *TERT*. The lentivirus construct included puromycin antibiotic resistance, thus, the cells were subjected to antibiotic selection, and surviving cells were expanded further. The over expression of *TERT* was confirmed by reverse transcription quantitative polymerase chain reaction (RT-qPCR) using a comparative ΔΔct method. The differences in the cycle thresholds for detection of TERT between primary cells and transduced cells showed increased expression of TERT in transduced cells. This increased expression however waned with increased cell passages. Encouragingly, the transduced cells were able to maintain similar morphology to primary cells over successive passages. The growth rate results also showed that primary cells' growth regressed with progressive passaging as that of transduced cells increased. Analysis of $TCID_{50}$/mL results for SAT and O serotypes of FMDV demonstrated no significant differences between primary and transduced cells. Virus isolation of SAT and O serotypes of FMDV gave similar time intervals to reach 100% cytopathic effect. These results demonstrate promise that the transduced cells could be used as a possible replacement for primary lamb kidney cells. Future work however must determine the extent of passaging that can be undertaken on transduced cells before there are notable changes to properties of the transduced cells. The transduced cells would also need to be validated using a broader range of virus serotypes and strain.

1. 0 Introduction

1.1 Background

Foot and mouth disease (FMD) is a disease that mainly affects cloven-hoofed animals of the order Artiodactyla such as cattle, goats, sheep, pigs, and 70 species of wild animals (Azeem, 2020; Cao et al., 2021). Foot and mouth disease is caused by a highly transmissible virus – foot and mouth disease virus (FMDV) - that has an extensive geographical prevalence, a diverse host range and is highly antigenically variable (Longjam et al., 2011). The FMDV disseminates swiftly via transmission routes, such as contaminated meat, milk, offal, skin, and non-sterile excretions, impacting multiple organ systems in infected animals (Dede et al., 2025). According to Casey-Bryars et al., (2018), US$2.3 billion is lost annually due to direct and indirect costs that are related to FMD in Africa. The Department of Veterinary Services in Botswana reports to have spent US$ 9,3 million between 2009-2014 on the control of FMD (Letebele, 2018). While the country strives to control the spread of the disease, beef production which accounts for approximately 80% of the agriculture sector's revenue experiences periodic losses as a result of FMD outbreaks (Kabelo et al., 2023). Given the quick dissemination of FMDV and the significant economic repercussions that may result from an outbreak, timely, precise, and specific laboratory diagnosis and identification of the serotype of the viruses implicated in disease outbreaks is imperative in order to mitigate the effects of FMD in vulnerable livestock populations (Howson et al., 2018; Jamal & Belsham, 2013; Wong et al., 2020).

A suspected case of FMD can variably be identified through the observation of clinical signs. The severity of symptoms in animals is influenced by various parameters, including species, age, viral strains, exposure dose to foot-and mouth disease virus (FMDV), and host immunity. In most adult animals, FMD is typically associated with a spectrum of morbidity. The disease however can be severe in juvenile animals such as young calves and lambs resulting in mortality (Guessoum et al., 2024). Animals infected with FMDV typically exhibit lesions on the tongue, muzzle, oral cavity, coronary cushions, and teats. The symptoms are typically more pronounced in cattle and highly raised pigs than in goats (Wong et al., 2020). In sheep, the detection using clinical signs is challenging due to the absence of conspicuous symptoms and these can swiftly lead to outbreaks (Guessoum et al., 2024). Furthermore, diagnosis predicated on clinical symptoms alone can be markedly incorrect, as other diseases such as swine vesicular disease (SVD), vesicular stomatitis, Seneca valley virus and vesicular exanthema exhibit symptoms analogous to FMD.

Therefore, confirmatory laboratory diagnosis of any suspected FMD case is essential for the management of an FMD outbreak (Wong et al., 2020; Zhang et al., 2018).

Foot and mouth disease is caused by a single-stranded, positive-sense, icosahedral, ribonucleic acid (RNA) virus known as foot and mouth disease virus (FMDV). FMDV belongs to the genus *Aphthovirus* and family Picornaviridae. The FMDV virion is a spherical icosahedron of approximately 25 to 30 nm in diameter (Dill & Eschbaumer, 2020). The genome can be functionally classified into three primary regions: (a) the 5' noncoding regulatory region, (b) the polyprotein encoding region further separated into L, P1, P2, and P3, and (c) the 3' noncoding modulatory region. The viral genome is translated into a singular polyprotein, which is subsequently cleaved by viral proteases into four structural proteins VP1, VP2, VP3, and VP4 and multiple non-structural proteins (NSPs) (L, 2A, 2B, 2C, 3A, 3B, 3C, and 3D). The non-structural proteins also regulate RNA replication, protein folding, virus assembly, regulation of viral replication and host cell functions (Kabir et al., 2024; Longjam et al., 2011). The P1 region of the genome encodes the four structural proteins VP1, VP2, VP3, and VP4, which are encoded by the 1D, 1B, 1C, and 1A genomic areas, respectively. The structural proteins constitute the icosahedral capsid, and are also accountable for the antigenicity and immunogenicity of the virus, since they encompass several antigenic sites recognised by neutralising antibodies (Kabir et al., 2024).

Foot and mouth disease virus has seven (7) immunologically distinct serotypes namely, Southern African Territories (SAT) 1, 2, 3, Asia 1, A, O, and C (*The Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 2022)*. Presently, only six serotypes are in circulation (A, O, Asia1, SAT1, SAT2, and SAT3), while serotype C is regarded as "extinct" (Medina & Segundo, 2024). Foot and mouth disease virus is categorised into seven global virus pools and is considered an endemic affliction in certain locations globally (Figure 1). FMD impacts vast territories in Africa, Asia, and South America. The geographical distribution of each FMD serotype differs, with serotype O exhibiting the broadest range (Guessoum et al., 2024). The cumulative incidence of FMD serotypes indicates that six of the seven serotypes; O, A, C, SAT1, SAT2, SAT3 have been reported in Africa, whereas Asia has encountered four serotypes (O, A, C, Asia1), and South America has recorded only three (O, A, C). Occasionally, serotypes SAT1 and SAT2 have been reported to invade the Middle East from Africa (Kabir et al., 2024; Maree et al., 2014). The FMDV serotypes are highly variable, immunologically distinct, and provide no cross immunity to one another.

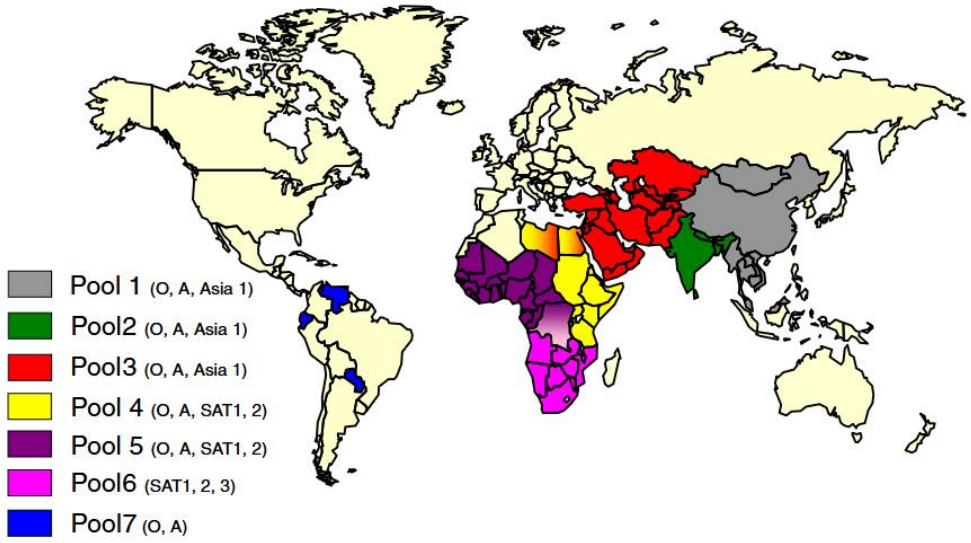


Figure 1: Global distribution of FMD with geographic hotspots of FMDV serotypes (Jamal & Belsham, 2013)

The different pools of FMDV that group the serotypes distribution globally. The pools are however not limited to the serotypes shown here as there can be introduction of exotic serotypes.

Foot and mouth disease virus demonstrates significant genetic and antigenic variation, complicating its eradication and prevention. The antigenic variability of FMDV is primarily dictated by the VP1 protein, which facilitates viral attachment and entry, confers protective immunity, and determines serotype specificity (Kabir et al., 2024). Antigenic variability is also ascribable to the evolution of the virus as it has an elevated mutation rate, brief generation time, substantial population size, and varied host range (Woldemariyam et al., 2023). The genetic diversity of the virus is also influenced by persistent infections and recombination processes which result in genomic heterogeneity (Dede et al., 2025). Animals with persistent infections can be clinically indistinguishable and serve as reservoirs for viral recombination. These reservoirs of naturally existing viral populations may, in certain instances, consist of a viral swarm or quasispecies, facilitating rapid adaptation to fluctuating conditions (Litz et al., 2025). Persistent infections contribute significantly to viral evolution by helping the virus to adapt to host immune response while the virus broadens its host range. Foot and mouth disease virus frequently engages in genetic exchange through recombination among several serotypes (Kabir et al., 2024). Viral recombination is the genetic exchange process occurring between distinct strains or serotypes of FMDV. Recombination can occur within host cells during co-infections with various FMDV strains, constituting a prevalent and crucial aspect of FMDV evolution. Recombination can influence the capsid-coding area, which dictates

the antigenic characteristics and host range of the virus (Kabir et al., 2024). Genetic alterations of the virus lead to emergence of new viruses that are genetically variable and immunologically distinct from the existing viruses which in turn complicates eradication and control of the virus.

Measures to eradicate FMDV are hindered by the restricted cross-reactivity between newly developing field strains and existing FMD vaccine strains (Litz et al., 2025). An existing vaccine candidate cannot be used for a new outbreak without vaccine matching. Effective management of field outbreaks necessitates that the vaccines employed are antigenically compatible with the prevalent field FMD viruses and the compatibility is established through vaccine matching (Eltahir et al., 2024). Selection of FMD vaccine candidates is challenged by the extensive genetic and antigenic heterogeneity of FMDV and the ongoing development of novel mutants from populations that evade the host immune response (Maree et al., 2014). Due to minimal cross-protection among serotypes, alignment between field and vaccine strains is essential for efficient FMD control programs. This necessitates the development of new strain-specific vaccines by adapting field viruses for cell culture propagation when the need arises (Kabir et al., 2024).

Laboratory-based diagnostic assays are a crucial aspect of the control and elimination of FMD as they facilitate precise confirmation of clinical observations. Diagnostic testing identifies FMD viral antigens, viral nucleic acids, or virus-specific antibodies in clinical samples. Established reference standards comprise viral isolation (VI), reverse transcription polymerase chain reaction (RT-PCR), enzyme-linked immunosorbent assays (ELISA), and virus neutralisation test (VNT) (Tan & Ward, 2024). Two main determinative laboratory test methods are suggested for diagnosis of FMD: virus isolation in cell culture followed by virus typing either by: (i) antigen enzyme-linked immunosorbent assay (AgELISA), (ii) virus genome (RNA) identification by reverse transcriptase polymerase chain reaction (RT-PCR) platforms and sequencing platforms. When virus concentrations are too low to be detected by AgELISA, or RT-PCR, clinical samples from animals suspected to have FMDV can be propagated in susceptible cell cultures through virus isolation to increase viral titres (Jamal & Belsham, 2013; *The Manual of Diagnostic Tests and Vaccines for Terrestrial Animals,* 2022*)*.

Virus isolation using cell cultures thus facilitates the study of viral morphology, increasing of virus titres, as well as the detection and characterisation of viral characteristics. The infection of cells with the virus will lead to changes in the morphology and biochemistry of the host cell which ensue from viral replication. These alterations which lead to cell destruction are called cytopathic effect (CPE) and confirm

the presence of a virus. The alterations can take on many different forms, including syncytium development, cell rounding, or cell lysis (Dolskiy et al., 2020; Fana et al., 2021). Virus isolation is the sole *in vitro* method to verify the presence of live FMDV however it has notable challenges such as; (1) handling infectious FMDV poses a considerable biosafety hazard; (2) cell cultures diminish in susceptibility to the virus over time and lose permissiveness to the virus through successive passages; (3) antibodies in samples from infected animals may entirely or partially neutralise FMDV; (4) virus isolation is significantly less analytically sensitive than RT-PCR (5) cytopathic effects can arise from other viruses, not solely FMDV, necessitating confirmation of positive results through alternative methods. False negatives can also rise at the beginning of infection in a VI test due to low virus titres. Virus propagation in cell culture also requires technical expertise, suitable laboratory facilities, and a great investment of time (Poonsuk et al., 2018). Despite these challenges VI is a crucial aspect of FMDV diagnosis.

The VNT assay is a serological test that can be used to confirm positive VI test results. VNT is used to detect the presence of antibodies that prevent infectivity of a virus (Gauger & Vincent, 2014). The VNT measures the titre of neutralizing antibodies post-exposure or post-vaccination. VNTs are performed *in vitro* and are based on the inhibition of virus infectivity in cell culture in the presence of neutralizing antibodies in serum (Tekleghiorghis et al., 2014). The presence or absence of cytopathic effect or evidence of viral infection using an immune reactive technique is used for titre determination. Cells that are susceptible to infection with the virus are crucial for VNT as the obtained virus titres are used to determine a relationship coefficient that is essential in determining a match or suitability of the vaccine strain and the field isolate (vaccine matching) (Tekleghiorghis et al., 2014). VNT tests are prone to variability due to the use of different cell batches when using primary cells (Jamal & Belsham, 2013). Standardising the source of cells is a crucial aspect as it can offer consistency when conducting VNT tests.

Virus isolation and VNT for FMD diagnosis both require the use of cells that are susceptible to infection with FMDV. The virus utilises host cellular apparatus to reproduce and for allied infection events (Gaboiphiwe et al., 2024; Han et al., 2018). Foot and mouth disease virus relies on cell receptors, primarily integrin family receptor molecules, to infect host cells. Integrin molecules function as receptors for several viruses, including field isolates of FMDV. Integrins are heterodimers composed of alpha and beta subunits, each existing in many forms. Six or seven unique integrin variants are identified as interacting with the conserved RGD amino acid motif found on the VP1 capsid protein of FMDV. These receptors are positioned on the superficial area of susceptible cells and facilitate the virus's attachment prior to the

commencement of the infection process (Li et al., 2021). The most common receptors used by FMDV on cultured cells and in susceptible animals are $\alpha_v\beta_6$, but other αv-integrins that are used include; $\alpha_v\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_8$ (Li et al., 2021; Wang et al., 2015).These integrins are mostly expressed in epithelial cells, as such epithelial cells are more sensitive to FMD infection. Cell systems with poor distribution of appropriate receptors exhibit a problem with either infection susceptibility or permissivity and consequently result in poor utility of the cells for the required diagnostic purpose (Brehm et al., 2009).

The Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, (2022) endorses bovine, ovine, and porcine cells for use in diagnostic assays including virus neutralisation tests and viral isolation. According to literature, primary cells that are used for FMDV propagation include primary lamb kidney cells, bovine thyroid cells, as well as the bovine, porcine and ovine palatine tonsils and dorsal soft palate cells (Hägglund et al., 2020; Kabelo et al., 2020; Mao et al., 2018; Sarry et al., 2023, 2024). In Botswana, primary lamb kidney cells are commonly utilised for diagnostic assays at the World Organisation for Animal Health Reference Laboratory for Foot and Mouth Disease (WOAH-RLFMD), (Fana et al., 2021; Gaboiphiwe et al., 2024; Kabelo et al., 2020; Teye et al., 2019). Despite the general sensitivity of primary lamb kidney cells to FMDV infection with different serotypes, the caveat in their utility for routine FMD diagnosis lies within the short-lived nature of the cells and the variation in the sensitivity caused by different cell batches (Gray et al., 2021; Kabelo et al., 2020).

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1.2 Problem statement

FMDV diagnosis by virus isolation and/or VNT relies on the use of cells. These cells can be primary cells that are sourced, prepared and cultured on per need basis or banked stable cell lines that are revived when required. The use of primary cells leads to variation of results in diagnostic tests such as virus isolation and VNT due to the varying degrees of sensitivity in cell batches of primary cells. A study conducted by Kabelo et al., (2020) reports a scenario where two batches of cells of the same primary cells showed different responses to viral infection with the same viruses. Commonly used stable cell lines such as BHK-21 are available to offer a cell culture alternative that has a longer life span. Unfortunately, their major setback is their decreased sensitivity to some FMDV serotypes such as the Southern African Territories (SAT) serotypes of FMDV which are predominant in the sub-Saharan Africa region (Kabelo et al., 2020). Cells with low sensitivity to FMDV have the potential to give false negative results. This can affect the time taken to release test results needed to make decisions necessary for prompt responses in case of an FMD outbreak. If FMD is not timeously and sensitively diagnosed livestock farmers will be affected since they rely on animals for draft power, income, milk, meat, hides, and socio-psychological support. Stakeholders in livestock or animal product trade will also be affected because of FMD-related transboundary movements and trade restrictions. Lamb kidney primary cells have been shown to be very sensitive to SAT serotypes of FMDV. Thus, the immortalization of lamb kidney primary cells for development of a cell line can offer a cell culture system that is more sustainable, sensitive, cheaper and resilient for FMD diagnostic testing. Such a cell line can have high diagnostic utility, facilitating prompt generation of results necessary to reinforce FMD control measures and limit economic repercussions.

1.3 General aim

To develop and characterise a lamb kidney cell line for *in-vitro* propagation of FMDV.

1.4 Specific objectives

To immortalise primary lamb kidney cells and determine the evolution of their properties over successive passages.

To validate the utility of the developed cell line by assessing its sensitivity to infection with different FMDV serotypes.

1.5 Hypothesis

Primary lamb kidney cells can be immortalised to create a highly sensitive, stable cell line for routine FMD diagnosis.

1.6 Expected outcomes

A cell line that is highly sensitive to FMDV that serves as a resource for consistent, routine FMD diagnosis. The cell line could potentially also be used for FMD vaccine production.

1.7 Justification of the study

Primary lamb kidney cells are routinely used for diagnostic tests such as virus neutralization tests and virus isolation (Fana et al., 2021; Kabelo et al., 2020; Lawrence et al., 2013). Primary cells have been shown to be very sensitive to SAT serotypes of FMDV, however the sensitivity of primary cells is subject to the batch of cells used at the time and the serotype of the virus that is used for inoculation of the cells (Kabelo et al., 2020; Kamal et al., 2014). Sensitive and timeous diagnosis of FMD can benefit from development of cellular tools such as the establishment of a cell line from primary cells of known excellent sensitivity to infection with FMDV. Primary lamb kidney cells are most routinely used for FMD diagnosis at the World Organization for Animal Health Reference Laboratory for Foot and Mouth Disease (WOAH-RLFMD), in Gaborone, Botswana. The sensitivity of the primary lamb kidney cells to SAT serotype viruses that circulate in Botswana and neighbouring countries can be leveraged for better diagnostic applications. Primary lamb kidney cells can thus be immortalised and then evaluated for utility in routine FMD diagnosis. The stable cell line would be advantageous because the homogenous cells would maintain reproducible high susceptibility to FMDV infection while bringing forth longevity of cell use, and practicality of the immortalised cells being banked for future use.

2. Literature review

2.1 Introduction

Immortalised cell lines are altered cells capable of indefinite proliferation. Immortalised cells exhibit genetic and phenotypic similarities to the parental cells while being capable of continuous culture (Chalak et al., 2024). The development of an immortalised cell line was initiated by George Otto Gey in the1950s. Gey, the leader of tissue culture research at Johns Hopkins University, initiated the process of establishing a non-aging cell line known as HeLa. HeLa cells were derived from a rare adenocarcinoma located in the cervix of a young lady named Henrietta Lacks (Chalak et al., 2024). Gey revealed that Henrietta's cells exhibited an unprecedented capability: they could be sustained and proliferated *ex vivo*, whereas cells derived from other sources typically survived for only a few days *ex vivo* (Khan, 2011). Therein, the fundamentals of cell immortalisation were established.

Immortality in cells can happen spontaneously in nature when cells are infected by tumour viruses such as; simian virus 40 (SV 40) and herpes simplex virus or by intentional introduction of longevity-associated genes, such as TERT (Martini et al., 2007; Maurisse et al., 2010; Soice & Johnston, 2021; Zhang et al., 2016). Additionally, immortalisation of cells can happen when antibody-producing B cells are fused with cancerous cells such as myeloma cells to create immortal hybrid cells called hybridomas (Mitra & Tomar, 2021). Both these methods can help the cells bypass cell senescence mechanisms and achieve continuous culture *in vitro*. As a way of avoiding senescence, cells can deactivate protein 53(p53) and the retinoblastoma (RB) tumour suppressor pathways. This dysregulation can happen due to tumour viruses or other environmental factors such as UV light and cigarette smoke. When cells lose cell cycle checkpoint pathways such as p53, protein 16 (p16), and retinoblastoma (Rb), they replicate continuously without dying (Cooper, 2000). In a cell cycle, the activation of p53 cells is induced by DNA damage and other cell stress-related problems, causing cell cycle arrest until the cell determines if the DNA is repairable. If the damage is deemed irreparable, p53 activates cell cycle arrest and eventually triggers apoptosis (Soice & Johnston, 2021). Activation of p16 and Rb follows, which will stop other proteins from triggering DNA replication, resulting in cell senescence. When cells have lost tumour suppressor pathways through inactivation of p53, p16, and Rb by simian virus 40 large T antigen (SV40LT) or other tumour viruses, they gain the ability to bypass senescence-mediated growth arrest and ultimately extend cellular lifespan. This method induces tumorigenesis in cells, which can potentially distort the genotypes and result in karyotype abnormalities and non-typical physiological phenotypes (Segeritz & Vallier, 2017).

Senescence in cells can also be avoided through evading the shortening of the telomeres which happens as chromosomes are replicated during cell division (Nicholls et al., 2012; Nogueira et al., 2021). Telomerase is an enzyme that preserves telomere length. This enzyme forms a substantial ribonucleoprotein complex responsible for the synthesis of telomeric DNA repeats in the 5' to 3' orientation. Telomerase comprises two distinct subunits: the catalytic component known as telomerase reverse transcriptase (TERT), encoded by the *TERT* gene, and the RNA component referred to as telomerase RNA component *(*TERC or TR), encoded by the *TERC* gene (Chalak et al., 2024). TERT as a catalytic subunit of telomerase, plays an important role in the regulation of telomerase. Telomerase is important for telomere maintenance, it protects telomeres from exonucleases and ligases thereby conserving them from deterioration or fusion (Chebel et al., 2009; Schellnegger et al., 2024). In somatic cells, chromosome telomeres shorten with every replication of DNA. This shortening of chromosome ends exposes them to damage due to the inability of cells to replicate the extreme ends of chromosomes. When telomeres are critically short, cells exit the cell cycle and undergo cell senescence (Nicholls et al., 2012). Telomerase activity is prominently expressed in germline cells, in immortalised cells, and in cancer cells, but it is poorly expressed in most somatic cells (Chebel et al., 2009; Mattson et al., 2013). The over-expression of *TERT* in somatic cells can allow them to have extended proliferative capacity (Soice & Johnston, 2021). The concept of TERT immortalisation involves transcending replicative senescence to result in cells that exhibit characteristics, aside from senescence, akin to their wild-type progenitors. Numerous criteria and assessments are commonly employed for evaluation of this phenotype. Immortalised cells must maintain a normal karyotype, lack malignant, tumorigenic traits, and exhibit comparable division rates and characteristics to the normal parental cells. Furthermore, if cells achieve immortality solely through *TERT* stimulation, they must have variations in *TERT* activity compared to their progenitors, acquiring immortality through this mechanism rather than through any oncogenic alterations (Shitova et al., 2024).

TERT is commonly overexpressed in the development of stable cell lines. Human TERT (*hTERT*) has been overexpressed in bovine thyroid cells by Mao et al., (2018) for investigating FMDV, to develop a cell line from mesenchymal stem cells derived from foetal porcine pancreas by Cao et al., (2011) and also to establish a foetal cow skin fibroblast cell line by Ren et al., (2022). However, there are instances where researchers developed cell lines using species-specific *TERT* such as in the isolation of porcine *TERT* to immortalise porcine fibroblasts (He et al., 2016), chicken *TERT* to immortalise chicken preadipocytes (

Wang et al., 2017), mouse *TERT* to immortalise mouse cells (Steele et al., 2010), and bovine *TERT* also to immortalise bovine foetal fibroblasts (Zhang et al., 2021). The use of bovine *TERT* to immortalise bovine cells was found to be more efficient than using *hTERT* as the telomeres of bovine *TERT* cells were reported to last longer than those of *hTERT* immortalised cells (Zhang et al., 2021). *TERT* over expression through the use with *hTERT* or species-specific expression is a crucial aspect of stable cell line development.

2.2 The use of lentiviral transduction to develop cell lines

Transduction is the use of viral means to deliver genetic materials to the target cells. It is an effective technique for introducing desired transgenes into target cells. Transduction utilises a viral vector to transfer the required nucleic acid material into the genome of host cells (Nayerossadat et al., 2012). Viruses such as lentiviruses, adenoviruses (type 2 and 5), and adeno-associated viruses can be used for transduction (Nayerossadat et al., 2012). Virus transduction can happen naturally through infection by tumour viruses or through using engineered viruses such as lentiviral to transfer genetic material (Elegheert et al., 2018). Lentiviral vectors are among the most efficient gene delivery systems, facilitating steady, long-term transgene expression in both proliferating and non-proliferating cells (Lei et al., 2010). The expressed nucleic acid becomes part of the cell genome, and it is replicated in daughter cells. Stable transduction is used for large-scale protein production, research into long-term gene regulation, and the generation of stable cell lines (Fus-Kujawa et al., 2021).

Lentiviruses are viral particles that have a lipid envelope and consist of two identical copies of linear single-stranded RNA genomes (Denning et al., 2013). The lentiviral vectors are widely used due to their great adaptability, efficient transduction, and ability to target a wide range of cellular and tissue types, including challenging-to-transfect cells like neurons and glial cells (Denning et al., 2013). Lentiviruses depend on the active transportation of the pre-integration complex via the nucleopore by the nuclear import machinery of the target cell. Lentiviruses possess the ability to infect both actively dividing and non-dividing cells, which renders them highly desirable for a range of gene delivery purposes (Denning et al., 2013). The transfer of exogenous *TERT* to cells using lentiviruses as delivery vehicles can effectively activate cell telomerase, leading to the elongation of telomeres, maintenance of chromosomal stability, enabling cells to progress through the metaphase 1(M1) and metaphase 2 (M2), which leads to

indefinite division and proliferation. Consequently, this process stably immortalises cells *in vitro* (Wang et al., 2022).

2.3 Selection of transduced cells

Nucleic acids delivering constructs such as lentiviruses usually contain selectable markers such as the green fluorescent protein (GFP) and antibiotic selectable marker genes (SMG) (Vain et al., 1998). The markers permit the selection of transduced cells (Fus-Kujawa et al., 2021). GFP has emerged as an essential instrument for the identification of gene expression in both prokaryotes and eukaryotes. GFP has been extensively utilised to elucidate spatial and temporal patterns of gene expression *in vivo* (Lissemore et al., 2000). GFP is easily identified in live, frozen, or fixed cells and is recognised with excellent sensitivity and specificity in suspended cells via flow cytometry. However, GFP may lose sensitivity during handling or expression may vary depending on the cell population (Swenson et al., 2007). Alternatively, antibiotic selectable marker genes can be used to select cells after transduction because they impart resistance to antibiotics which are introduced at an appropriate concentration into the culture medium. Consequently, only the cells that have successfully acquired and expressed the selectable marker genes can neutralise the antibiotic, thereby surviving and proliferating *in vitro* on the designated selection medium (Súnico et al., 2024). Most of the selectable marker genes are NeoR, BsdR, HygR, BleoR, and PuroR genes, which confer resistance to antibiotics such as geneticin (G418), blasticidin, hygromycin B, zeocin, and puromycin respectively (Guo et al., 2021). Their reliability and ease of use have made antibiotic selectable markers indispensable in cell line establishment.

2.4 Confirmation of successful transduction

Following transduction, it is important to confirm if indeed the gene of interest has been integrated successfully in the cells. During transduction the lentiviral DNA is integrated randomly into the host cells, therefore there is a possibility of unintentionally incorporating target genes at non-target sites but rather at any other homologous locus within the cell (Petrov et al., 2022). Incorporation of genes at non-target sites can unfortunately lead to altered expression of the gene of interest. Gene expression is thus determined quantitatively and phenotypically.

Elevated levels of *TERT* can be quantitatively confirmed using the total RNA of the cells. Overexpression of the gene of interest may be confirmed by tests such as reverse transcription quantitative polymerase chain reaction (RT- qPCR), Western blotting, immunofluorescence (immunohistochemical detection), and

in some cases telomeric repeat amplification protocol (TRAP) assay (Steele et al., 2010; Sun et al., 2022). These tests may be repeated as the cell line passages progress to determine if the gene of interest is still expressed. Reverse transcription quantitative PCR integrates Polymerase chain reaction (PCR) with fluorescent reporter chemistry to monitor template amplification with enhanced sensitivity and specificity (Harshitha & Arunraj, 2021). Reverse transcription quantitative PCR enables real-time observation of PCR progression. Polymerase chain reaction is an enzymatic technique employed *in vitro* to amplify a specific DNA sequence exponentially, producing thousands to millions of copies of that segment. The required reagents comprise template DNA, primers, probe, and thermostable DNA polymerase (Artika et al., 2022). To facilitate accurate analysis, pure RNA undergoes a crucial preliminary step of reverse transcription, when RNA molecules are transformed into cDNA by the enzyme reverse transcriptase (Đermić et al., 2023). The probe is non-extendable because it is fluorescently labelled at its 5' end and has a quencher at the 3' end, while the primers are extendable (Arya et al., 2005). At the annealing stage, both the primer and the probe bind to the template, the primer will then be extended from the upstream end region of the template by the nuclease activity of the polymerase until it reaches the region of the probe. When the probe is still intact, fluorescent emissions from the dye are absorbed by the quencher (Arya et al., 2005). The nuclease activity of the polymerase will cleave the probe releasing the dye from the proximity of the quencher. This will lead to fluorescence emission; additional cleavage removes the probe allowing the primers to extend until the end of the complementary DNA (cDNA). As more cleavage happens more dye is released giving more fluorescence until detection above a minimum is achieved called a threshold cycle (Ct value). Ct values are defined as the fractional PCR cycle number at which the report fluorescence is greater than the minimum detection level (Arya et al., 2005). Ct values are said to be directly proportional to the amount of starting RT-qPCR product, it takes fewer cycles to achieve a cycle threshold with a higher template, than with fewer templates. Reverse transcription quantitative PCR is extensively used to identify and measure many forms of transcripts, including messenger RNA, ribosomal RNA, and non-coding RNA. Besides enhanced accuracy, sensitivity, and speed, a primary advantage of RT-qPCR over conventional PCR is its ability to establish a dependable quantification relationship between the initial target sequence count (prior to PCR amplification) and the quantity of amplicon generated in a specific PCR cycle. This capability is crucial for the precise quantification of target nucleic acids, which is essential for mRNA quantification in gene expression analysis in laboratory specimens (Arya et al., 2005).

Normalizing genes or housekeeping genes are usually used as endogenous controls in RT- qPCR assays to standardise the amount of starting material in the expression of genes under investigation. Normalization in RT-qPCR is the process of adjusting for variations in sample quantity, RNA quality, and reaction efficiency to ensure accurate and reliable quantification of gene expression. Normalizing genes are used with the assumption that the genes remain consistently expressed in the tissues or cells under investigation and are conserved across species. Endogenous reference genes are considered optimum since the detection and amplification of both reference and target genes occur under identical conditions (Herath et al., 2020). Normalizing genes include; glyceraldehyde 3-phosphate dehydrogenase (GAPDH), β-actin, β2-microglobulin, porphobilinogen deaminase, and the transferrin receptor, with GAPDH being the most commonly used (Barber et al., 2005). Normalisation is essential for the comparison of samples from diverse sources that may possess differing amounts of mRNA. Variations in the amount of mRNA may arise from discrepancies in the effectiveness of mRNA extraction and isolation processes. The variation in the starting quantity of mRNA for RT-qPCR reactions can in turn result in experimental variability (Herath et al., 2020). Therefore, normalisation enhances the reliability of relative RT-qPCR investigations (Adeola, 2018).

Post confirmation of expression of the gene of interest, the cells are phenotypically characterised. Characterising the cells is essential to ascertain whether they are immortalised while maintain the morphology and growth rate of their progenitors. The morphology, size, and characteristics of immortalised (transduced) cells must remain consistent and closely mirror those of the progenitor cells. This is usually done by approximation under a light microscope (Shitova et al., 2024). In the process, the viability, and the ability to form a confluent monolayer is assessed. Parent cells are utilised as a control by culturing them concurrently with immortalised cells for ongoing comparison. The process is also used to assess whether the cells can survive beyond their primary counterparts as a way of monitoring *TERT* expression beyond transduction in developed cell lines. This is crucial since overexpression of *TERT* is done with the intention to immortalise cells and maintain consistent growth rates that are unimpeded by cellular senescence (Shitova et al., 2024).

After development, stable or continuous cell lines can be passaged over 100 times before attaining replicative senescence, without morphological modifications that could compromise the reliability and repeatability of cell culture-based diagnostic results (Gray et al., 2020). Stable cell lines have been developed and utilised for FMD diagnosis. These include; baby hamster kidney (BHK-21), swine kidney

(IB-RS-2), foetal goat cell line (ZZ-R 127), foetal porcine kidney cells (LFBKαVB6), bovine kidney (BK) cells, the human telomerase reverse transcriptase bovine thyroid (hTERT-BTY) and porcine kidney derived PK-15, and SK 6 (Brehm et al., 2009; Gray et al., 2021; Kamal et al., 2014; Mao et al., 2018). The cell lines were developed mainly to circumvent the short life span of primary cells, and also to attain sensitive cell culture systems to targeted FMDV serotypes (Mao et al., 2018).

Most of the different cell lines available to propagate FMDV are not validated for SAT serotypes of FMDV which are prevalent in the Southern African region (Gaboiphiwe et al., 2024). The most commonly used FMDV culture systems in Botswana are the primary lamb kidney cells for diagnostic tests and the BHK-21 cell line for vaccine production (Fana et al., 2021; Kabelo et al., 2020; Teye et al., 2019). The deficient utility of primary cells for diagnostic purposes has already been previously outlined. The BHK-21 cell line also poses some challenges for propagation of SAT serotypes of FMDV because the cell line is permissive but not sensitive to FMDV serotypes such as SATs (Burman et al., 2006; Dill & Eschbaumer, 2020; Gaboiphiwe et al., 2024; Kabelo et al., 2020). It is thus imperative that there be concerted effort towards development of cell lines that are suitable for propagation of SAT serotypes of FMDV. Such efforts would be invaluable for extensive study of SAT viruses and region-specific disease control.

3.0 Materials and methods

3.1 Kill curve determination

Primary lamb kidney (RM) from a 6-month-old lamb at passage 1 were collected from liquid nitrogen storage, thawed and transferred to lamb kidney growth media (RM media), consisting of Minimum Essential Medium (MEM) (Thermo Fisher, United States of America), 5% adult bovine serum (Gibco, New Zealand), 10 000 U/mL penicillin/ 20 mg/mL streptomycin (Gibco, United States of America), 2.5% lactalbumin hydrolysate (Gibco, Austria), and sodium bicarbonate (Sigma Aldrich, United States of America). The pH of the media was maintained between 7.4 and 7.6 by adjusting with 1N hydrochloric acid (Carlo EBA reagents, Italy). The cells were numerated by introducing 1 mL of the cell's suspension to 2 mL of 2.5% trypsin (Sigma Aldrich, United states of America). The mixture was incubated at 37 °C for 5 minutes, thereafter 1 mL of trypsin and cells mixture was introduced to 2 mL trypan blue before loading into a haemocytometer for numeration. The cells were reconstituted to a density of 0.15×10^6 cells/mL using RM media, and a 200 µL volume was used to seed Nunc 96 well micro-culture plates (Thermo Fisher Scientific, United states of America), (passage 2). The cells were incubated at 37 °C in a 5% CO_2 incubator (Thermo Fisher Scientific, Jouan, United States of America) for 72 hours. A stock solution of 1 mg/ml of puromycin dihydrochloride (Glentham Life Science, Germany) was used to prepare different dilutions of puromycin - 0 µg/mL, 1 µg/mL, 2 µg/mL, 3 µg/mL, 4 µg/mL, 5 µg/mL, 6 µg/mL, 7 µg/mL, 8 µg/mL, 9 µg/mL, 10 µg/mL, and 11 µg/mL. When the cells were approximately 90% confluent, the spent RM media in the Nunc 96 well micro-culture plates (Thermo Fisher Scientific, United states of America), was changed to media with different concentrations of puromycin dihydrochloride (Glentham Life Science, Germany); 0 µg/mL, 1 µg/mL, 2 µg/mL, 3 µg/mL, 4 µg/mL, 5 µg/mL, 6 µg/mL, 7 µg/mL, 8 µg/mL, 9 µg/mL, 10 µg/mL, and 11 µg/mL to determine the kill curve of the primary cells. The plates were monitored daily with a light microscope (Olympus, Model: CKX 31, Hachioji, Tokyo), for the deterioration of cells as they are affected by puromycin dihydrochloride (Glentham Life Science, Germany). The lowest concentration of puromycin that killed the cells within 10 days was determined to be used for selecting transduced cells.

3.2 Transduction of primary cells

Three kinds of media were prepared for this stage;

1. RM media as described in 3.1 above

2. Media without serum and antibiotic consisting of Minimum Essential Medium (MEM) (Thermo Fisher, United States of America), 2.5% lactalbumin hydrolysate (Gibco, Austria), and sodium bicarbonate (Sigma Aldrich, United States of America) at pH 7.4-7.6. This media was used as transducing media.

3. Media without antibiotic consisting of MEM (Thermo Fisher Scientific, United States of America), 5% adult bovine serum (Gibco, New Zealand) 2.5% lactalbumin hydrolysate (Gibco, Austria), and sodium bicarbonate (Sigma Aldrich, United States of America), at pH 7.4-7.6. This media was used as selection media. The absence of antibiotics allowed for addition of different puromycin working concentrations.

RM cells at passage 3 were cultured in Nunc 96 well micro - culture plates (Thermo Fisher Scientific, United states of America) at a cell density of 0.15×10^6 cells/mL in RM media and incubated at 37 °C in a 5% CO_2 incubator to achieve 70% confluency in 24 hours. After 24 hours, the spent media was discarded and replaced with 200 µL of transduction media consisting of;

1. cationic transduction reagent -TransduceIT (Mirus™, United states of America) at a concentration of 5 µg/mL
2. recombinant lentiviral supernatant (Cellomics CMV, GFP, PURO), United States of America) at 1 µg/mL.

Four wells were reserved as negative and positive controls for antibiotic selection.

The cells were incubated at 37 °C in a 5% CO_2 incubator (Thermo Scientific, Jouan) for 24 hours. When 24 hours had elapsed, transduction media was discarded, and replaced with fresh RM media for 48 hours (Figure 2). At the end of the 48 hours incubation period, the RM media in the transduced cells and the positive control was replaced with selection media containing puromycin at 1 µg/mL for 3 days, while two well of the negative control were maintained with RM media. The cells in the positive control were used to track the effects of the antibiotic on the cells, after successful selection of transduced cells, selection media was replaced with normal RM media. The confluency of surviving cells after antibiotic selection was assessed visually through a light microscope (Olympus, Model: CKX 31, Hachioji, Tokyo) and expressed as a percentage of the starting confluency of the cells. The cells were allowed to grow back to confluency in RM media. The media in individual wells was changed every 72 hours to remove cell debris and dead cells to allow surviving cells to grow back to confluency (Passage 4) (Figure 2). Transduction of the primary cells was conducted in duplicate.

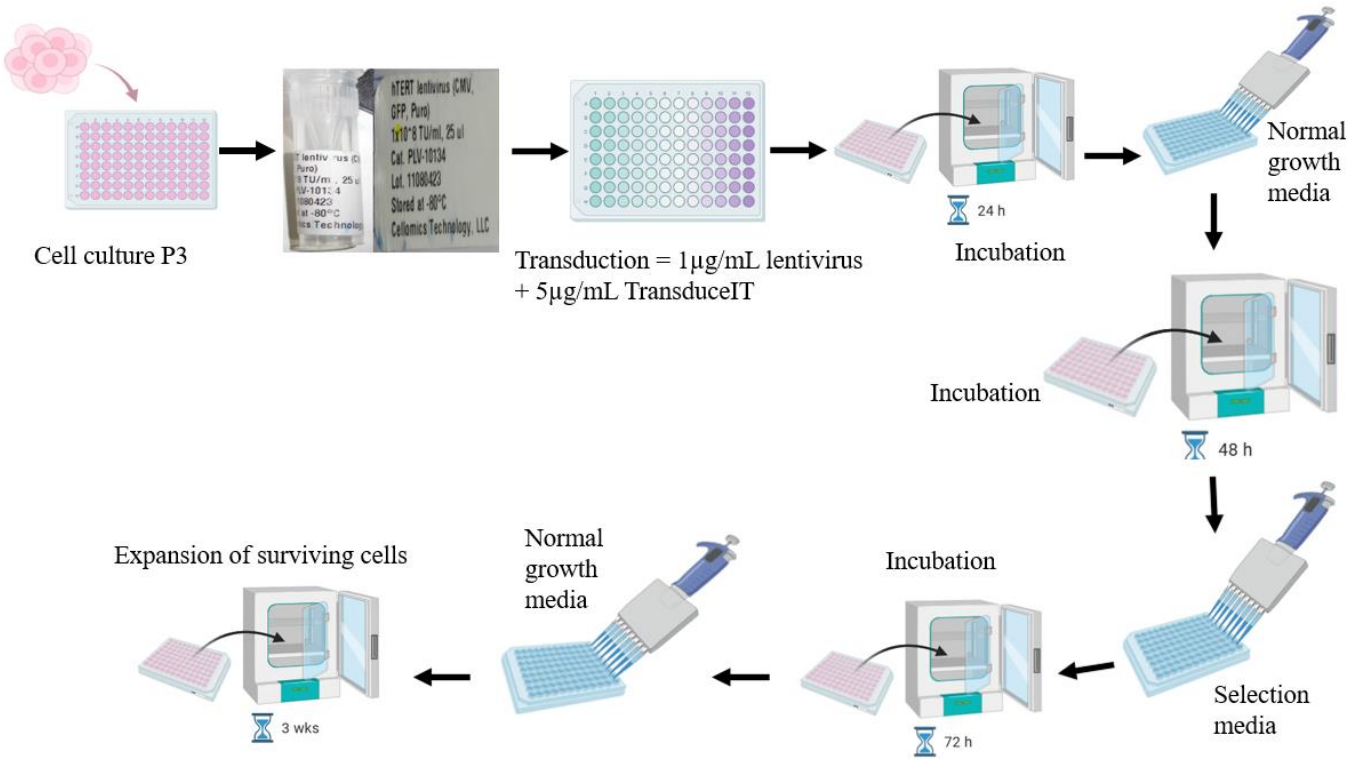


Figure 2: Illustration of the transduction process

The cells were transduced at passage 3. After incubation for 24 hours, the transducing media was removed and changed to normal growth media. The cells were incubated further for 48 hours then selected with media containing puromycin dihydrochloride for 72 hours. After selection, normal growth media was reintroduced for expansion of surviving cells.

Upon achieving 50-60% confluency of surviving cells in 96 well plates, the cells were reintroduced to selection media for 24 hours to confirm their puromycin resistance. After survival was confirmed through growth of cells in selection media, the surviving cells were cultured in RM media and allowed to go back into their actively dividing stage. Thereafter the transduced cells were trypsinated in 100 µL of RM media consisting of 1% trypsin (Sigma Aldrich, United States of America), for 10-15 minutes at 37º C incubated in a 5% CO_2 incubated in a Thermo Scientific incubator (Jouan, United States of America) to completely detach the cells from the plate. The trypsin activity was stopped by adding 100 µL of serum (Gibco, New Zealand) to each well and incubated at 23ºC for 5-10 minutes. The cells from 96 well plates (Nunc MicroWell Thermo Fisher, United States of America) were collected from each well using a Finnpipette®1000 µL micropipette and tips (Thermo Fisher United Kingdom) into a 15 mL Eppendorf

tube (Eppendorf, Germany) and centrifuged (Eppendorf, 5901Ri, Germany), at a speed of 500 × g at 4ºC for 10 minutes to remove the media. The transduced cells were reconstituted in 15 mL RM media.

3.3 Confirmation of cells transduction

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) was conducted on the transduced cells and the primary cells to confirm successful TERT transduction.

3.3.1 RNA extraction

RNA from both transduced cells and primary cells was extracted using the Quick-RNA™ Miniprep kit (Zymo Research, United States of America). About 1 mL of the transduced cells or the primary cell culture was pelleted by centrifugation at the speed of 500 × g and temperature of 4ºC for 1 minute. The supernatant was removed, and the pellet resuspended in 300 µL RNA lysis buffer and incubated for 10 minutes at 23ºC. The sample was centrifuged at 16,000 × g and 4ºC for 30 seconds using a MicroCL R17 centrifuge (Thermo Fisher Scientific, United States of America) to remove the particulates. The supernatant was transferred to a nuclease-free tube and then into a Spin-Away™ filter with a collecting tube for centrifugation (Thermo Fisher, MicroCL R17, United States of America) at 16 000 × g and 4ºC for 30 seconds. The flowthrough was saved, and an equal volume of 99.9% ethanol was added to it and mixed by vortexing to ensure through mixing. The mixture was transferred into a Zymo-Spin™ IIICG column and centrifuged (ThermoFisher, MicroCL R17, United States of America) at 16,000 × g and 4ºC for 30 seconds. The samples were further subjected to DNAse treatment by washing in 400 µL wash buffer followed by centrifugation (Thermo Fisher, MicroCL R17, United States of America) at16 000 × g and 4 ºC for 30 seconds. After washing, 5 µL of DNase 1 (1U/µL) and 75 µL of DNA digestion buffer were mixed and added directly into the column matrix. The column matrix was left to incubate for 30 minutes at room temperature. After the incubation, 400 µL of RNA prep buffer was added to the column before centrifugation (Thermo Fisher Scientific, MicroCL R17, United States of America) at 16,000 × g and 4ºC for 30 seconds. The flowthrough was discarded, and the column was washed using 700 µL of RNA wash buffer. The flow through from the washing was discarded, and the column was rewashed with 400 µL of RNA wash buffer and centrifuged at 16,000 × g and 4ºC for 1 minute to ensure complete removal of the wash buffer. The 100 µL of DNase free water was then added to the column matrix and centrifuged at 16,000 × g and 4 ºC to elute the RNA. The quantity and the quality of the RNA eluted was determined using a Nanodrop (Thermo Fisher Scientific, United States America) before storage at -20ºC until needed.

3.3.2 RT-qPCR

One step quantitative RT- qPCR was conducted using the constituents below for hTERT/ GAPDH;

Table 1: Constituents of hTERT /GAPDH RT- qPCR mix.

Reagent Name	**Quantity**
RT-qPCR reaction mix	10 µL
RNase free water	5.0 µL
10 µM hTERT/GAPDH forward Primer hTERT F – 5' GCATTGGAATCAGACAGCAC 3' GAPDH F - 5' ATGGGTGTGAACCATGAGAA 3'	0.5 µL
10 µM hTERT/GAPDH reverse primer hTERT R- 5' CCACGACGTAGTCCATGTTC 3' GAPDH R- 5' GTGCTAAGCAGTTGGTGGTG 3'	0.5 µL
5 µM probes hTERT Probe 5' 6-FAM CGCCCTGCTGACGTCCAGA 3' BHQ-1™ GAPDH probe 5' 6-FAM CCTCAAGATCATCAGCAATGCCTCC 3' BHQ-1™	0.5 µL
RNA polymerase	1 µL
RNA Sample	2.5 µL
Total	**20µL**

The RT-qPCR reaction was conducted using the 7500 Real-Time PCR System (Applied Biosystems, United States of America) under the following conditions; reverse transcription for 10 minutes at 55°C, thermal cycling steps were as follows: initial denaturation at 95°C for 60 seconds , denaturation at 95°C for 15 seconds , annealing at 60ºC for 60 seconds, extension at 72 °C for 60 seconds, all the cycling steps were repeated for 40 cycles before final extension at 72 °C for 300 seconds.

The reaction was repeated in triplicate for 3 samples from passage 4, 10, and 18 of transduced cells and passages 3, 5, 7 of primary cells. The RT-qPCR was conducted to generate Ct values to quantify and compare the amount of hTERT in the cells. GADPH was used as a housekeeping gene to normalize the quantity of the gene of interest.

3.4 Continuous passaging of primary and transduced cells

3.4.1 Passaging of primary cells

The cells from passage 1 were also seeded into 150 cm^2 (T-150) cell culture flask (Corning Life Sciences, United States of America) at a cell density of 0.15×10^6 cells/mL diluted in 100 mL media and incubated in a 5% CO2 incubator for 72 hours until 100% confluence (passage 2). At 100% confluency, the cells were trypsinated using 15 mL of RM media consisting of 1% trypsin (Sigma Aldrich, United states of America). After addition of trypsination media the cells were incubated at 37 °C in a 5% CO_2 incubator (Thermo Fisher Scientific, Jouan, United States of America) for 10- 15 minutes. Trypsin activity was stopped by addition of 15 mL of bovine serum (Gibco, New Zealand) at 23ºC for 5-10 minutes, before centrifugation at the speed of 500 x g and temperature of 4ºC for 10 minutes to remove the supernatant. After centrifugation the cells were resuspended in 15 mL of RM media, 1 mL of the cell suspension was introduced to 2 mL of trypan blue before loading into a haemocytometer for numeration. Passage 3 of these cells was initiated by diluting cells to a concentration of 0.05×10^6 cells / mL in 200 mL of RM media to seed a 490 cm^2 roller bottle (passage 3), the cells were incubated at 37ºC in a roller incubator (Bellco Biotechnologies, United states of America), at 1 revolution in 3 minutes / 0.33 rounds per minute (RPM) for 120 hours. After these cells reached 100% confluency, they were trypsinated as stated above, and stored in cryopreservation media consisting of RM media as well as 7.5% dimethylsulfoxide (DMSO) (Sigma Alrich, Germany) and 10% adult bovine serum (Gibco, New Zealand). In preparation for cryopreservation, the cells were stored at -20ºC for 2 hours, -40ºC for 2 hours and -80ºC overnight, before transferred to liquid nitrogen for later experiments.

To monitor the confluency, morphology, viability and cell count per passage of primary cells, two T-25 25 cm^2 cell culture flasks (T-25) (Corning Life Sciences, United States of America) were seeded with primary cells using a starting concentration of 0.15×10^6 cells/ mL in 15 mL of RM media. This was repeated for passages 2 to 4 of primary cells. To seed passage 5 and 6, cell seeding density was adjusted to the 0.10×10^6 cells/ mL in 15 mL of RM media in T-25 culture flasks (Corning Life Sciences, United States of America) to standardise them to the count of transduced cells. Passage 7 and 8 were initiated with a seeding density of 0.06×10^6 cells/mL in 100 mL of RM in a T-150 cell culture flask (Corning Life

Sciences, United States of America). All cell cultures were incubated at 37°C in a 5% CO_2 incubator (Thermo Fisher Scientific, Jouan, United States of America) for 72-120 hours until 80-100% confluency.

At the end of each passage, the confluency and the morphology of the monolayers was determined before the cells were trypsinated using 15 mL of RM media consisting of 1% trypsin (Sigma Aldrich, United states of America). Upon addition of trypsination media the cells were incubated at 37°C in a 5% CO_2 incubator (Thermo Fisher Scientific, Jouan, United States of America) for 10- 15 minutes. Trypsin activity was stopped by addition of 15 mL of bovine serum (Gibco, New Zealand) at 23ºC for 5-10 minutes, before centrifugation at the speed of 500 x g and temperature of 4ºC for 10 minutes to remove the supernatant. After centrifugation the cells were resuspended in 15 mL of RM media, which was used for numeration, viability assessment and seeding of the next passage. To numerate cells at the end of each passage, 1 mL of the cell suspension was introduced to 2 mL of trypan blue before loading into a haemocytometer for numeration. The primary cells were passaged alongside the corresponding transduced cells as described. All remaining cells after seeding of each passage were centrifuged (Eppendorf, 5901Ri, Germany) at 4ºC and 500 × g for 10 minutes then reconstituted in 1 mL of RM media, with 10% adult bovine serum (Gibco, New Zealand) and 7.5% DMSO (Sigma Alrich, Germany) and cryopreserved as described above.

3.4.2 Passaging of transduced cells

The transduced cells were expanded from passage 4 after trypsination from the 96 well plates in 3.2 until passage 18 using RM media. The seeding density of each passage was largely dependent on the count from the previous passage as well as to allow for cryopreservation of the passage. The cells from passage 4 were seeded in a T-25 culture flasks (Corning Life Sciences, United States of America) for continuous passaging of transduced cells. The seeding density used to initiate passage 5 of the transduced cells was 0.09×10^6 cells/mL in 15 mL of RM media, these were cultured as a pool of cells (Passage 5) and allowed to grow at 37 °C in a 5% CO_2 incubator for 9 days (Thermo Scientific, Jouan United, States of America) (Figure 1). All the passages of the transduced cells had their confluency, morphology assessed, before trypsination, numeration, and viability assessment at the end of each passage as described in 3.4.1 above. The cells harvested at the end of passage 5, were diluted with 30 mL of RM media resulting in a seeding density of 0.15×10^6 cells/ml and distributed into two T-25 cell culture flasks (Corning Life Sciences, United States of America) to initiate passage 6. At the end of passage 6, a cell density of $0{,}09 \times 10^6$ cells/mL in 100 mL of RM media was used to reconstitute the cells and initiate passage 7 in a T-150

(Corning Life Sciences, United States of America). All the passages in T culture flasks were incubated at 37 °C in a 5% CO_2 incubator for 5-7 days to allow the development of a monolayer.

As the passages increased, the culture flasks size was also increased to accommodate the increasing number of transduced cells. Passage 8 was seeded in 490 cm^2 roller bottles at a cell density of 0,06 × 10^6 cells/mL in 200 mL of RM media, while for passage 9 the same cell density as passage 8 was maintained in 300 mL of RM media but the cells culture flask was changed to an 850 cm^2 roller bottle. For Passages 10-18 a seeding density of 0,10 × 10^6 cells/mL in 300 mL of RM media was maintained in 850 cm^2 roller bottles. The cells in roller bottles were all incubated at 37 °C in a roller incubator (Bellco Biotechnologies, United states of America), at a speed of 0.33 rounds per minute (RPM) for 120-168 hours. One 850 cm^2 from passage 10 was used for continuous passaging, while another bottle was used to seed an 850 cm^2 roller bottle to expand cells for validation of the transduced cells through virus isolation and $TCID_{50}$ of the transduced cells. One 850 cm^2 roller bottles for each transduction was maintained for continuous passaging purposes from passages 10-18 (Figure 3). The same seeding density was maintained for the roller bottles expanded for validation tests. All remaining cells after seeding of each passage were centrifuged (Eppendorf, 5901Ri, Germany) at 4 °C and 500 × g for 10 minutes then reconstituted in 1 mL of RM media with 10% adult bovine serum (Gibco, New Zealand) and 7.5% DMSO (Sigma Alrich, Germany) and cryopreserved as described in 3.4.1 above.

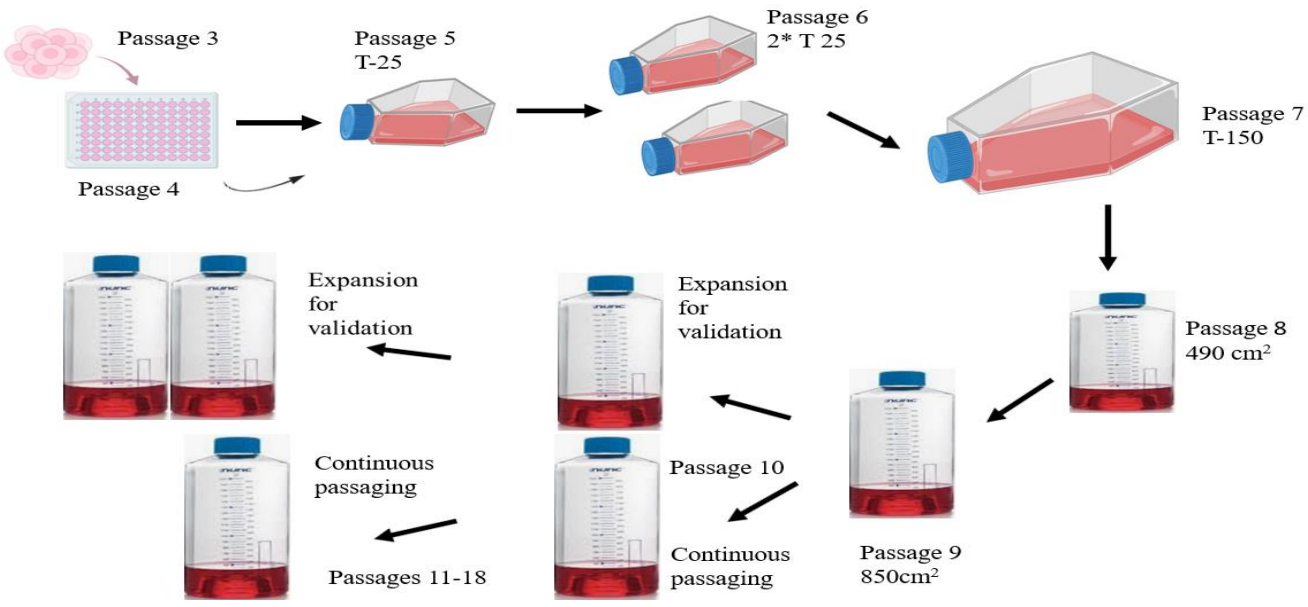


Figure 3: Illustration of the expansion of transduced cells.

Transduced cells were expanded from a 96 well plate until the cells were grown in 850 cm^2 roller bottles. Passages 4-6 were grown on T-25 Corning® cell culture flasks, passage 7 in a T-150 Corning® cell culture flask, Passage 8 in a 490 cm^2 roller bottle and from passage 9 upwards in 850 cm^2 roller bottles.

3.5 Characterization and validation of the transduced cells

3.5.1 Cell confluency, morphology assessment and viability assay

As the transduced cells were expanded, they were passaged alongside their primary cells until the monolayers reached 80-100% confluency. The cells were assessed for morphology and confluence formation using a light microscope (Olympus, Model: CKX 31, Hachioji, Tokyo). Cell viability was conducted using a trypan blue assay to assess the viability of the cells after every passage. Cell viability was determined by introducing 1 mL cells trypsinated at the end of each passage into 2 mL of trypan blue and loaded in a haemocytometer for renumeration. To determine viability, both dead and live cells were counted using a haemocytometer as described in 3.1 and 3.4 above. The percentage of live cells as a proportion of the total cells was used to determine viability.

3.5.2 $TCID_{50}$/mL assay

The 50% tissue culture infectious dose ($TCID_{50}$) was used to determine the sensitivity of cells to FMDV serotypes SAT 1, 2, 3, and O. Primary and transduced cells were cultured at a concentration of 0.15 x10^6 cells/mL in Nunc 96 well micro- culture plates (Thermo Fisher Scientific, United states of America), and allowed to grow to 90-100% confluence. The virus samples at 7.0 $TCID_{50}$/mL; SAT1/BOT05/23, SAT1/BOT08/20, SAT 109, SAT2/MAL05/22, SAT2/UGA11/17, SAT 251, SAT 2035, SAT2/ZIM05/18, SAT3/ZAM 08/17, SAT3/ZAM08/17, SAT 309, O/UGA/17 and O Manisa were serially diluted. One in ten serial dilutions ranging from log 10^{-1} to 10^{-11} were made. RM Media (2500 µL) was filled using pipettes into 11 haemolysis tubes (Jeulin, France) for each virus sample, then 250 µL of each virus sample inoculated in the first haemolysis tube (Jeulin, France), and serially diluted until the last haemolysis tube (Jeulin, France). 100 µL of serial dilutions from log 10^{-4} - 10^{-11} virus dilutions of FMD viruses were used to infect both the primary and transduced cells in 96 well plates. The plates were incubated at 37 °C in a 5% CO_2 Jouan incubator (Thermo Scientific, United states of America) for 72 hours. The presence/absence of CPE was determined after 72 hours of incubation, after which 50 µL of 10% citric acid (Sigma Alrich, Switzerland) was introduced in the plates and incubated at room temperature for 30 minutes and then stained with 100 µL of 5% crystal violet (Glentham Life Sciences, Germany) for another 30 minutes before interpretation. $TCID_{50}$ was conducted in duplicate for each virus

sample. Log $TCID_{50}$/mL was calculated using the Spearman-Karber method. The titres of each well were expressed as $TCID_{50}$/mL (Kabelo et al., 2020).

3.5.3 Virus isolation

The transduced and primary cells were characterized through virus isolation of FMDV serotypes SAT 1, 2, 3 and O. Virus isolation allows for FMDV to be cultured and examined *in vitro* typically in cell cultures to subsequently assess its biological, antigenic, and genetic characteristics. The samples used for virus isolation were; SAT1/BOT05/23, SAT1/BOT08/20, SAT 109, SAT2/MAL05/22, SAT2/UGA11/17, SAT 251, SAT 2035, SAT2/ZIM05/18, SAT3/ZAM 08/17, SAT3/ZAM08/17, SAT 309, O/UGA/17 and O Manisa. After determining the $TCID_{50}$/mL of each virus stock, all the virus samples were diluted and standardised to log 7.0 $TCID_{50}$/mL before inoculation for virus isolation. The primary and transduced cells were cultured in culture media made up of growth media (RM Media), consisting of Minimum Essential Medium (MEM) (Thermo Fisher, United States of America), 5% adult bovine serum (Gibco, New Zealand), 10 000 U/mL penicillin/ 20 mg/mL streptomycin (Gibco, United States of America), 2.5% lactalbumin hydrolysate (Gibco, Austria), and sodium bicarbonate (Sigma Aldrich, United States of America in T-25 cell culture flasks (Corning Life Sciences, United States of America) and incubated at 37 °C in a 5% CO_2 Jouan incubator (Thermo Fisher Scientific, United States of America). Cells were inoculated with 500 µL of each virus at 90-95% confluency and incubated at 37 °C in a 5% CO_2 Jouan incubator (Thermo Scientific, United States of America) for 1 hour to enable the FMDV, to infect the cells. After incubation the infected culture flasks were rinsed with 10 mL of RM media after incubation to remove any unabsorbed virus, after which each flask was filled with 15 mL of RM media for further incubation until 100% CPE was visualised. Virus isolation was conducted in triplicate for each viral sample. The effects of virus infection cells or CPE was monitored using a light microscope Olympus (Model: CKX 31, Hachioji, Tokyo after 12, 18 and 24 hrs (Fana et al., 2021).

3.6 Statistical analysis

$TCID_{50}$/mL was calculated using the Spearman-Karber method as;
$TCID_{50}$/mL= log (lowest dilution at which 100% CPE is observed) + I (logarithm interval between dilutions) × [0.5 – (total number of wells displaying CPE/total number of replicate wells per dilution)] (Lei et al., 2021).

The student T-test was used to determine any statistical variance between the mean log $TCID_{50}$/ mL results obtained using primary cells and the transduced cells.

The ratio of dead and living cells was determined through light microscopy by quantifying the quantity of stained (dead) and unstained (living) cells over the total number of cells utilising a Neubauer chamber (Avelar-Freitas et al., 2014).

Relative quantification was used to compare the expression of the TERT gene between the primary cells and the cell line using the $-2^{\Delta\Delta Ct}$ model and it was automatically calculated by the ABI 7500 machine using the formula:

$-2^{\Delta\Delta Ct}$ = ΔCt (target sample) -ΔCt (reference sample)

= ΔCt (target gene - normaliser gene)- ΔCt (reference gene - normaliser gene gene).

4.0 Results

4.1 Puromycin dihydrochloride kill curve determination for primary lamb kidney cells

Primary lamb kidney cells isolated from a 6-month-old lamb, were utilised at the 2nd passage to determine the kill curve of the primary cells using puromycin dihydrochloride. Kill curves are commonly employed to assess the impact of antimicrobials by monitoring the growth and death of cells across a broad range of antimicrobial concentrations (Foerster et al., 2016). Prior to initiating a transduction experiment aimed at developing a stable cell line, it is crucial to ascertain the appropriate dose of selective antibiotic by conducting a kill curve assessment. The kill curve precisely determines the optimal concentration of selective antibody required to eliminate untransfected cells within a selection period of 10 days. Primary cells were subjected to different concentrations of puromycin dihydrochloride ranging from 0-11 µL to determine the selecting concentration. The response of the cells to each concentration of the antibiotic was observed over a period of 3 days and the minimum concentration of puromycin dihydrochloride that could kill the primary lamb kidney cells in a period of 72 hours was determined to be 1 µL/mL as presented in Figure 4.

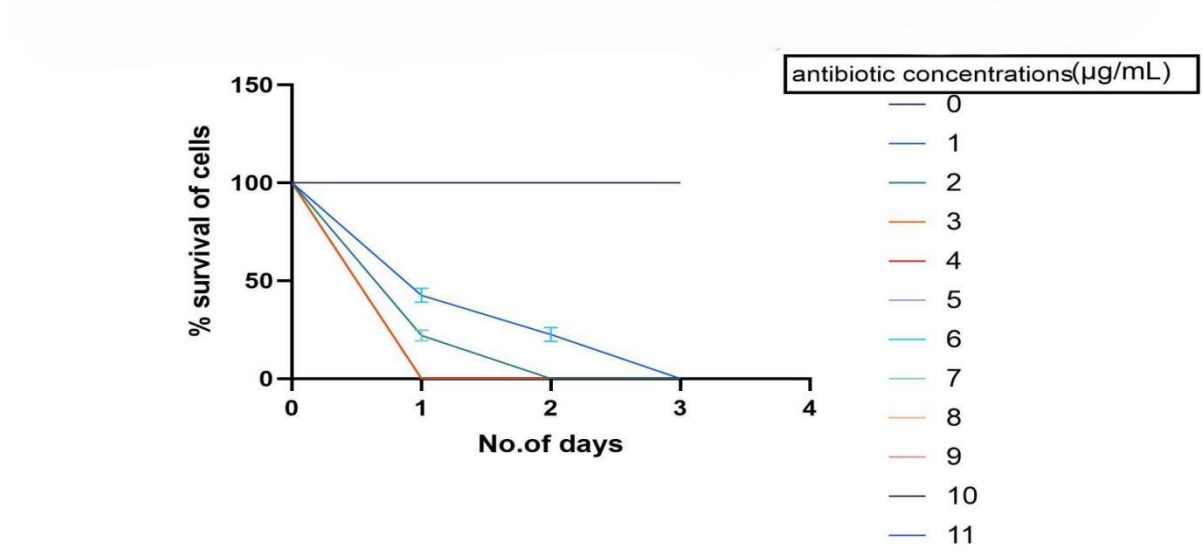


Figure 4: Effects of Puromycin dihydrochloride on primary lamb kidney cells

Treatment with 1–11 µL/mL puromycin dihydrochloride resulted in cell death as signified by cell rounding and detachment. A monolayer was visible up to 2 µL/mL on day 1 (~40% viability at 1 µL/mL; ~23% at 2 µL/mL). By day 2, only the control (0 µL/mL) remained fully confluent, while viability at 1 µL/mL declined to ~22%. All treated cells were dead and detached by day 3.

Primary lamb kidney cells showed signs of death such as rounding and detachment from the surface of the cell culture vessel when subjected to the different concentrations of puromycin dihydrochloride, except the control (0 µL/mL) control. On day 1, the culture media without antibiotic had a fully intact monolayer (100% confluency) while in 1 µL/mL concentration only approximately 40 % of cells were confluent. At a puromycin concentration of 2 µL/mL, cell confluency was about 23% while there was no observable cell confluency at puromycin concentrations of 3-11µL/mL on day 1. On day 2 of incubation, the control (no puromycin) remained fully confluent with an intact monolayer, while the culture with 1µL/mL concentration of puromycin showed further decrease in confluency to approximately 22%. At a puromycin concentration of 2 - 11 µL/mL all the cells had completely detached from the cell culture vessel surface on day 2. On day 3, the cell monolayer was visible only in the control culture (no puromycin) while the cells in lowest puromycin concentrations 1 µL/mL were detached from the cell culture vessel surface.

4.2 Transduction of primary cells

To achieve stable overexpression primary lamb kidney cells were transduced with a lentiviral vector encoding *TERT*. Transduction was enhanced with 5 µg/mL of TransduceIT reagent to reduce the high electrostatic repulsions between a negatively charged cell and an approaching wrapped virus (Denning et al., 2013; Storck et al., 2017). Based on the determined kill curve, the transduced cells were then selected with 1 µg/mL of puromycin dihydrochloride over 72 hours and grown up to 50/60% confluency over 3 weeks (Figure 4). Transduction efficiency based on antibiotic selection was estimated to be approximately 3% of the total cells per well.

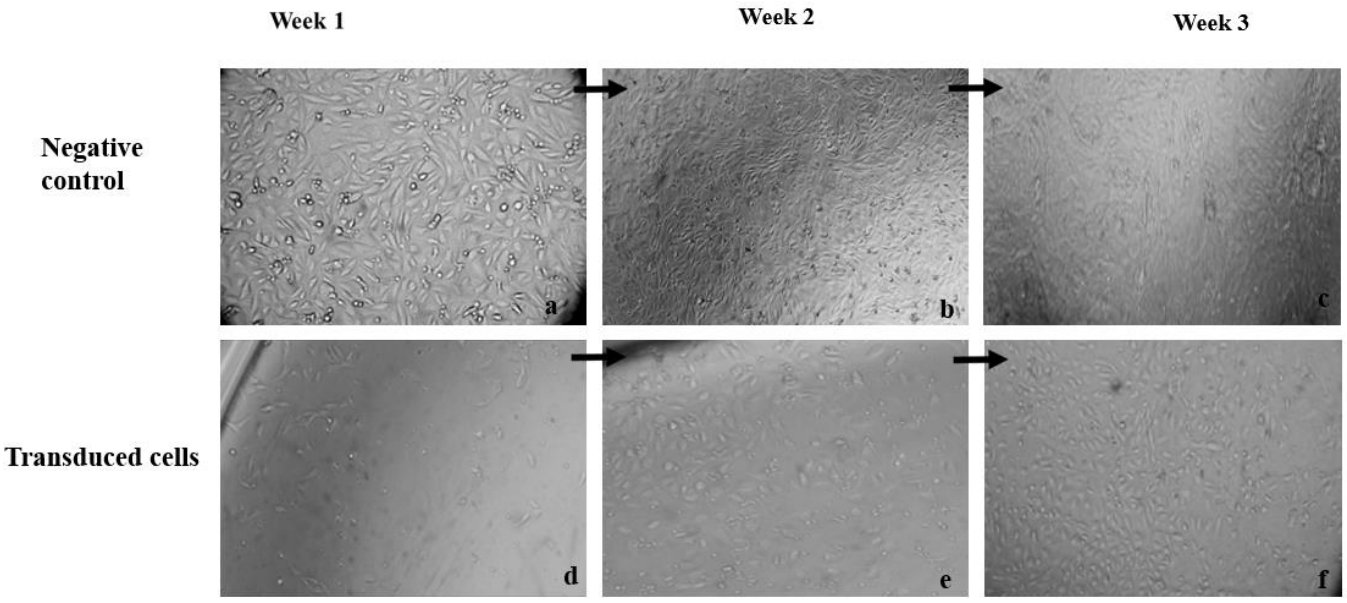


Figure 5: Visualisation of transduced cells after antibiotic selection.

Cells that survived puromycin selection were cultured in RM media at 37 °C in a 5% CO_2 incubator for three weeks. The growth of the cells was observed under a microscope under weekly observation. Cell confluency steadily increased over the 3 weeks period of incubation.

4.3 Confirmation of transduction using RT-qPCR

To confirm the transduction of the primary cells, analysis of *TERT* gene over expression was conducted through comparative $-2^{\Delta\Delta CT}$ RT-qPCR to determine the change fold of *TERT* gene expression in the transduced cells using the primary cells as a reference. Normalisation was performed using glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The $2^{-\Delta\Delta Ct}$ results were calculated automatically by the system by calculating the ΔCt of the target gene in the experimental sample - ΔCt of the target gene in the reference sample. The mRNA expression was characterised as a fold-change relative to the reference cells' value. Passage 4 of the transduced cells had a -15,31-fold change in its expression of TERT, P10 had a -11,4644-fold change in its expression of TERT, and lastly P18 had a -4,2141- fold change in the expression of TERT as reference to passage 3 of the primary cells (Figure 6). The results indicate that *TERT* was detectable only at a low passage number for primary cells. On the other hand, *TERT* expression was detected over 18 passages for transduced cells although the expression levels waned over time as shown on Figure 6.

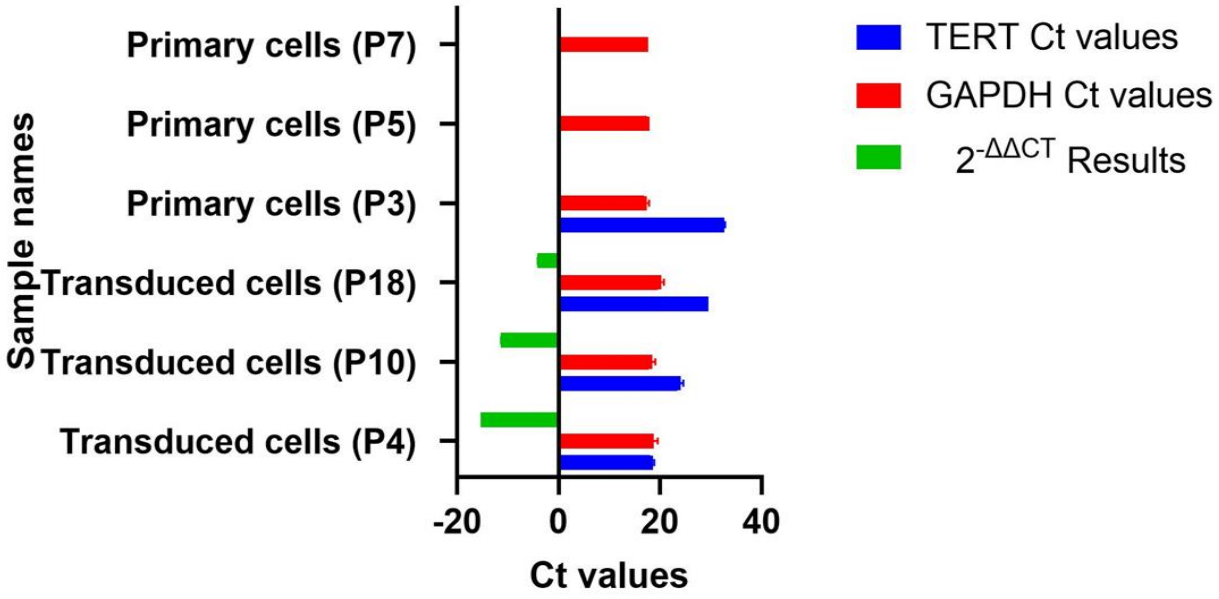


Figure 6: -$2^{\Delta\Delta CT}$ and Ct of different passages of transduced and primary cells

TERT expression levels were determined from the different levels of primary and transduced cells. GAPDH was used as a normalising gene. Passage 3 of the primary cells was the reference passage..

4.4 Expansion of primary and transduced cells

After the transduced cells were selected using puromycin dihydrochloride, the cells were trypsinated from a 96 well plate and transferred to T-25 flasks for expansion in RM media for the two transductions performed. The primary cells were cultured alongside the transduced cells in the absence of selecting antibiotic to monitor the effects of transduction on the cells. The transduced cells appeared in clumps after trypsination and seeding in a T-25 flask while primary cells were single cells, suggesting that transduced cells may have undergone cell stress caused by the transduction process. Growth in primary cells was visible in two days (Figure 7), after seeding while new growth was not visible in transduced cells T-25 flasks until day 4 (Figure 8). Although the initial cells count for both primary and transduced cells were standardised for expansion in T-25 plates, the transduced cells took more than a week to grow to 90-100% (Figure 8) while their primary counterparts were confluent at day 5 (Figure 7). These findings suggest that transduction has a temporary effect on the proliferation capacity of cells.

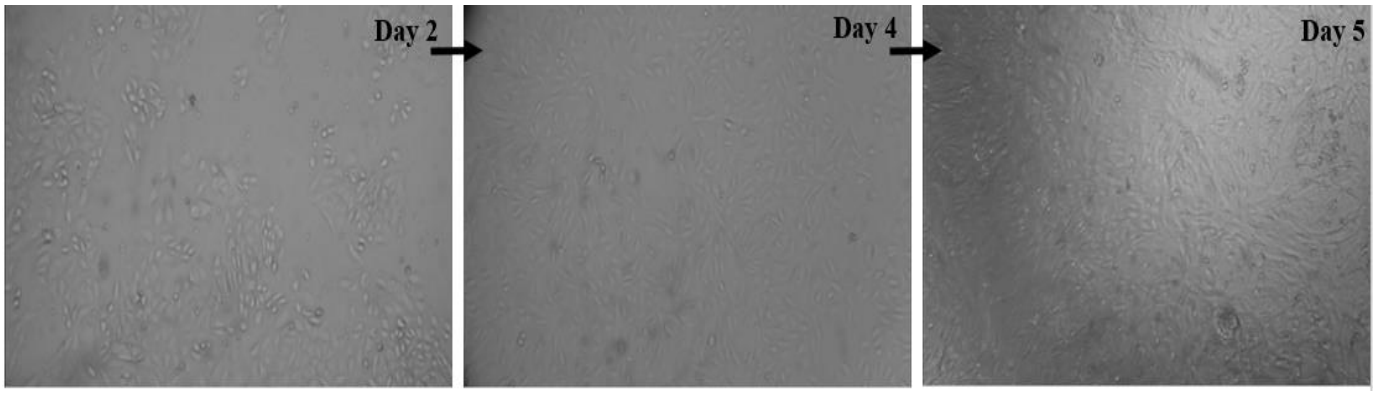


Figure 7: Confluence formation of primary cells after antibiotic selection

The primary cell populations during expansion were allowed to grow back to 100% confluency in T-25 flasks.

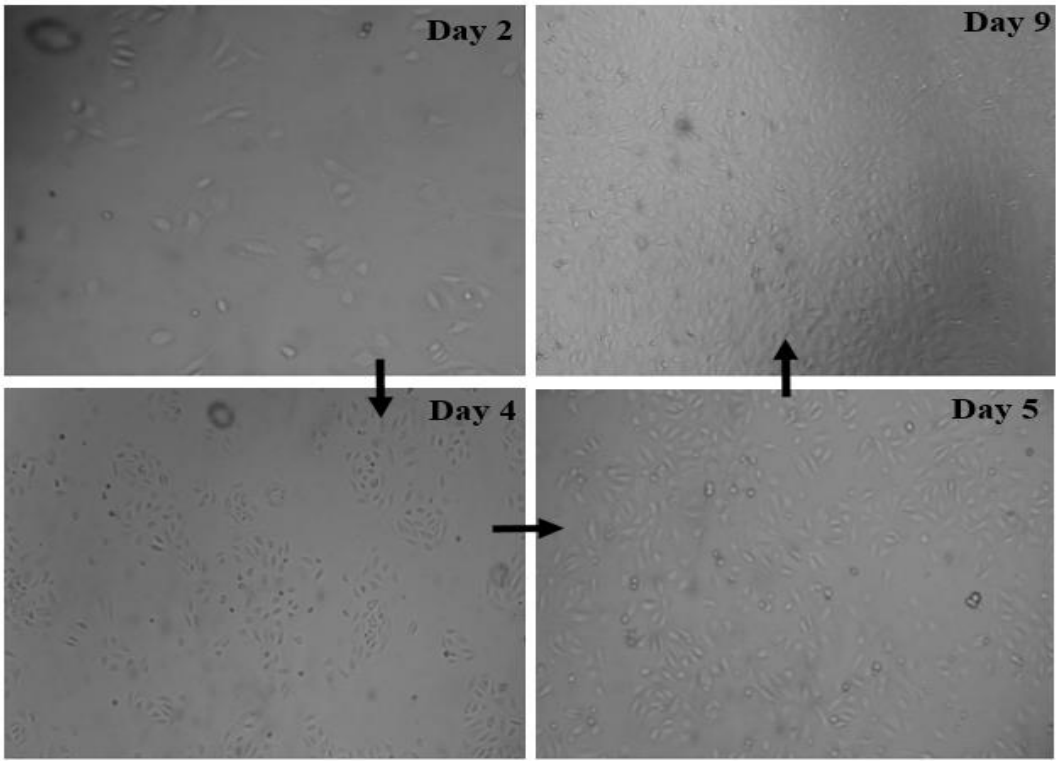


Figure 8: Confluence formation of transduced cells after antibiotic selection

The transduced cell populations were expanded and allowed to grow back to 80 - 100% confluency in T-25 flasks.

4.5 Morphology tracking of primary and transduced cells during passaging

Examining cellular morphology is the most straightforward and direct approach for assessing cellular health and stability. Cell morphology may differ based on cellular health and, in certain instances, the state of differentiation (*American Type Culture Collection, Technical Document*, 2022).The morphology of primary cells was tracked alongside the transduced cells from passage 2 until the primary cell culture ceased growing in passage 8 as shown in Figure 6. The density of the primary cells was standardised at the beginning of passage 5 to those of transduced cells in order to have comparable results at each passage. Morphology tracking was important to determine if transduction had any effect on the morphology of the cells. Both primary cells and transduced cells were a combination of spindle shaped or elongated structures and rounded cells. Both cell shapes were visualised in both populations of the cells, with an increase of rounded cells in the transduced cells. The transduced cells were highly proliferative suggesting cell cycle based morphological changes.

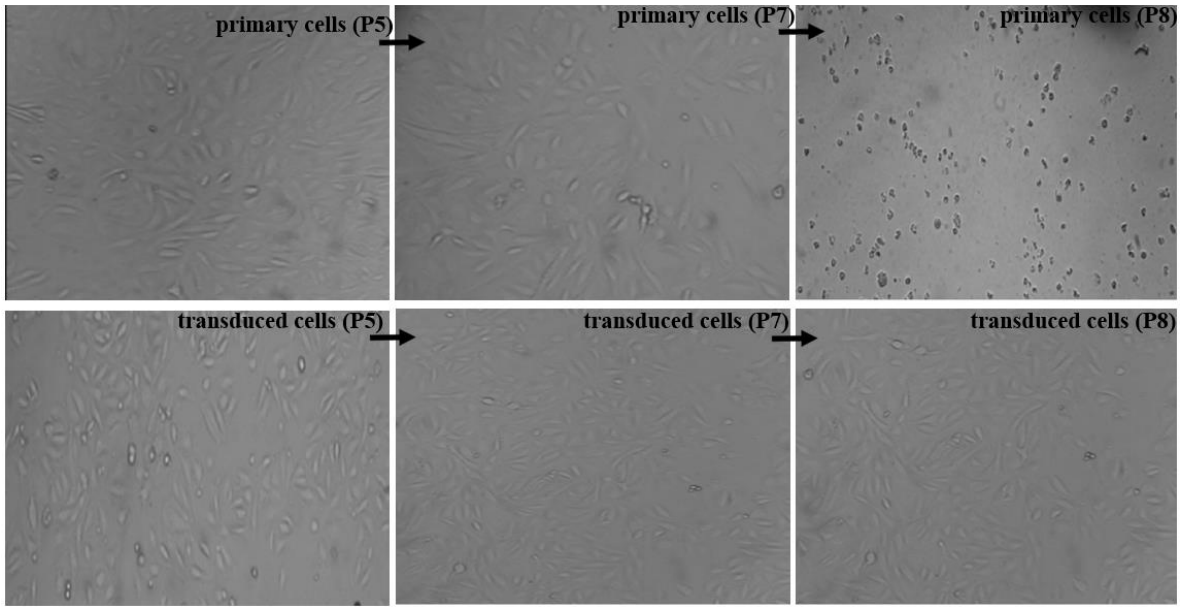


Figure 9: Morphology comparisons of primary and transduced cells

The morphology of primary cells (passages 5-8) and transduced cells from (passage 5-8).

4.5.1 Morphology tracking of transduced cells

Morphology tracking of transduced cells was continued in subsequent passages after the primary cells were no longer viable to assess if they maintained the same morphology as primary cells. It is well documented that cell phenotype can alter during passaging, potentially impacting the repeatability of in

vitro experimental outcomes (Handl et al., 2020). The transduced cells mostly appeared as a single layer (monolayer) of thin flattened cells. Morphology of the transduced cells was a combination of spindle shaped and rounded cells across all the passages from passages 10-18. Passage 14 exhibited the highest proportion of healthy, spindle-shaped cells and the fewest rounded cells.

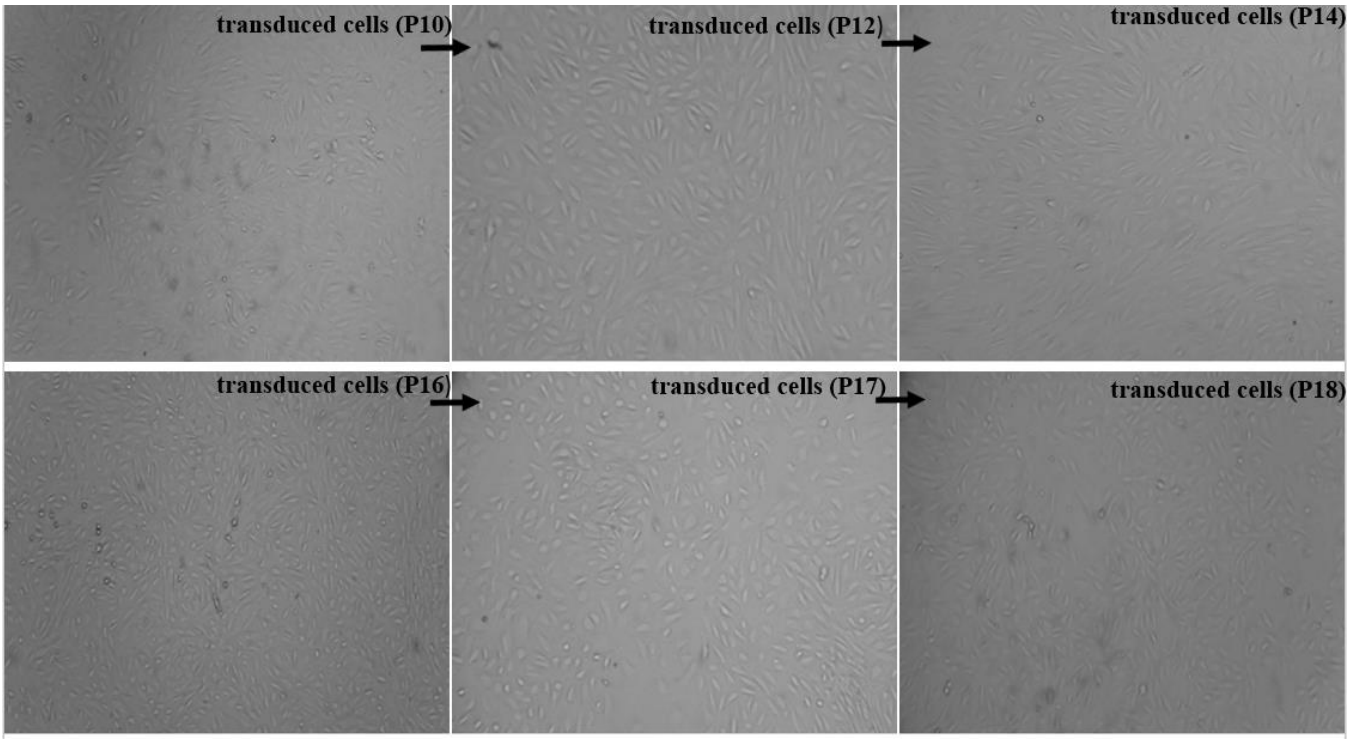


Figure 10: Morphology tracking of transduced cells passages 10-18

The morphology of the transduced cells tracked from passages 10-18 after the death of the primary cells. The cell morphology was closely monitored to assess any deviation from the morphology of primary cells.

4.6 Confluency, viability and cell numeration of primary and transduced

4.6.1 Confluency of primary and transduced cells

The confluency of the primary cells and the transduced cells were monitored to monitor the effects of transduction and successive passaging on the cells. It is important to monitor the confluency of both cell types since upon reaching confluency, these cells cease proliferation and begin to undergo apoptosis in the absence of sub-culturing (Ammerman et al., 2008). The confluency of the primary cell was tracked

from passage 2 until passage 7, passage 8 did not grow to confluence. The confluency of the transduced cells was tracked from passage 4, since transduction was done at Passage 3. The confluency of the cells as shown in Figure 8 ranged between 50-100%. The average confluency of the primary cells was 88.5% while that of transduced cells was 91%. The confluency of primary cells started decreasing at passage 5, as subsequent passages had lower confluences, likely due to increased cell death. During the initial passages (4-9) the transduced cells typically reached confluence after 7- 9 days of culture but from passage 10 onwards, the transduced cells reached 90-100% confluence in just 5 days. The shortened confluence time with increased passages suggest that cells underwent a post-transduction recovery or adaptation phase between passage 4 and 9. Transduction did not have much effect on the confluency of the cells as the cells could still achieve confluency above 90% up to passage 18.

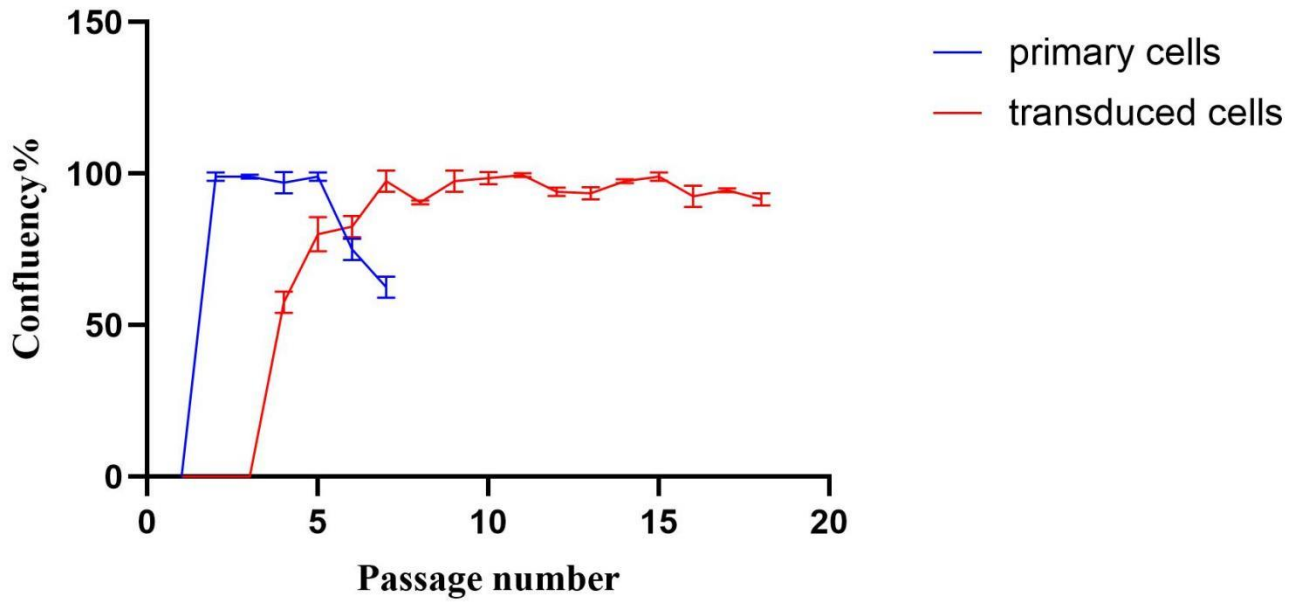


Figure 11: Confluency of primary and transduced cells

The confluency of the primary and transduced cells as observed from passage 2 -7 (primary cells) and passage 4-18 (transduced cells).

4.6.2 Viability of primary and transduced cells

The study of cell viability is a valuable tool after conducting a range of experimental processes such as trypsination (Avelar-Freitas et al., 2014) which is carried out each time that adherent cell cultures undergo passaging. Cell viability is based on the preservation of cell membrane integrity which is a fundamental factor in differentiating between dead and living cells. Consequently, dyes that have the ability to

specifically enter the cytoplasm of dead cells such as trypan blue are employed alongside trypsin. The viability of the primary and the transduced cells was determined after every passage using trypan blue staining. This was to determine the effects of transduction on cell viability as passaging progressed. This was also important to track if transduction had any significant impact on the viability of the cells. The viability of the primary cells was tracked from passages 2-7, and that of the transduced cells was done from passages 4-18 since the primary cells were transduced at passage 3. The results show that the initial passages of primary cells (2-3) were more viable than the transduced cells (Figure 9). After passage 3, the viability of the primary cells reduced gradually until the cells ceased growing. The viability of transduced cells was low after transduction, but the viability increased with the number of passages, and eventually the transduced cells viability stabilised to an average of 91% from passages 7-18. This finding was consistent with the confluency results described above, indicating that transduced cells underwent a recovery and adaptation phase between passage 5-7 post transduction.

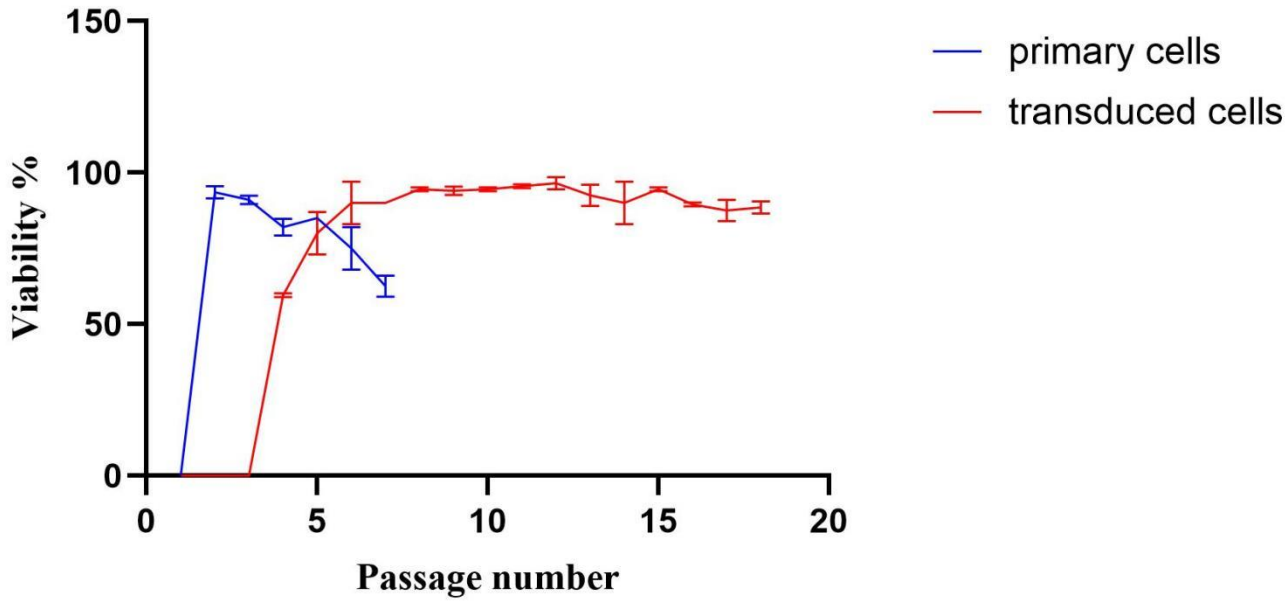


Figure 12: Viability of primary and transduced cells.

Viability of primary cells was observed from passage 2-7 while the viability of transduced cells was observed from passage 4-18. Viability assessed the integrity of the cells after trypsination during sub-culturing.

4.6. 3 Cell count per passage of primary and transduced cells

Assessing cell proliferation rates can provide significant insights into a cell culture's reaction to a stimulus. Conducting a growth curve analysis is essential for assessing culture stability and identifying several critical attributes, including the optimal timing for subculturing, ideal dilution, and projected plating efficiency at different cell densities (*American Type Culture Collection, Technical Document*, 2022). In this case it was important to assess if transduction had any effect on the growth rate of the cells. The number of living cells is intrinsically linked to growth rates, as the growth rate represents the positive aspect of the rate of change in population size. The number of cells for each passage was monitored at the end of each passage. As depicted in Figure 10, the cell counts of primary cells was initially higher than those of transduced cells, but the cell counts gradually decreased as passages increased. The transduced cells were initially lower in number, but the counts increased with each passage and eventually stabilised after passage 10 indicating that the cells have reached their maximum proliferation capacity. The primary cells proliferated up to passage 7 but passage 8 did not attach on the surface of the culture flask therefore assessment of growth kinetics was ceased at passage 7 for primary cells.

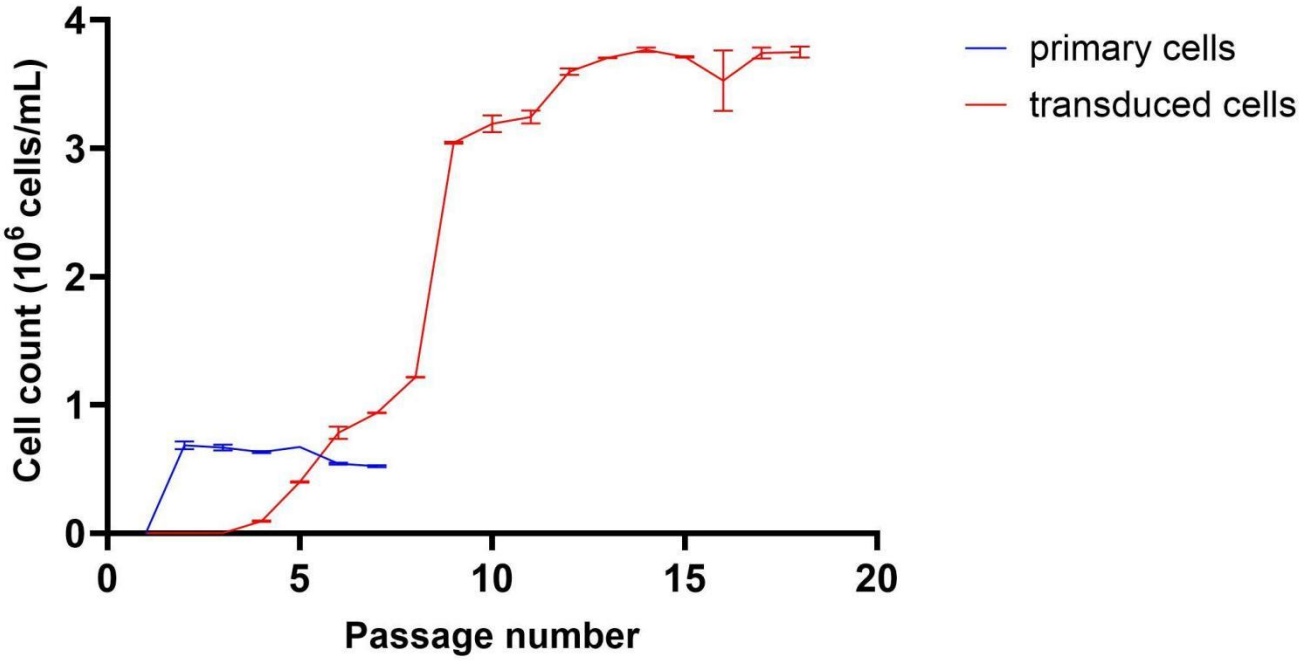


Figure 13: Cell counts per passage of primary and transduced cells.

The cell counts of primary cells observed from passage 2-7 and transduced cells observed from passage 4-18.

4.7 Assessment of the sensitivity of primary and transduced cells to virus infection using tissue culture infectious dose (LogTCID$_{50}$/mL)

TCID$_{50}$ uses end-point dilutions to ascertain the dilution at which 50% of the inoculated cell culture wells exhibit infection. The virus sample is serially diluted in logarithmic increments of log10, and wells with a specified confluent cell layer are infected with each dilution of the virus. Following an incubation period, dictated by the duration required for the virus to reproduce and trigger cell death, all inoculation cell culture wells are evaluated for the presence of virus-induced cytopathic effects (CPE) (Hochdorfer et al., 2022). CPE is the observable changes in cell morphology resulting from viral replication and serves as a crucial diagnostic characteristic in virology. These effects may encompass cell rounding, syncytia formation, and cell lysis, varying according to the virus and the cell type employed (Bastawecy et al., 2025). The purpose of TCID$_{50}$ was to determine/compare the sensitivity of primary cells and transduced cells to infection with different serotypes. TCID$_{50}$ was performed using passage 14 of the transduced cells and passage 2 of the primary cells (Figure 11). Passage 14 of the transduced cells was among the passages where the transduced cells had started to have high and consistent growth rates, and these passages were targeted for characterisation. Passage 2 of the primary cells is the most commonly used passage for TCID$_{50}$. A student-t test was conducted on the log TCID$_{50}$/mL results obtained from the primary and transduced cells to assess the effect of transduction on the cells. The log TCID$_{50}$/mL test results between the primary cells and transduced cells were considered significantly different if p- value < 0.05 and not significant if p-value > 0.05. Primary cells viral titres were slightly above the viral titres of transduced cells except for SAT2/UGA11/17, SAT2/ZIM05/18, SAT3/ZAM08/17, SAT3/ZAM08/20 and O Manisa as shown in Figure 14. The O Manisa depicted the highest titres of all samples. Higher TCID$_{50}$ indicates the virus can easily access the cells and make more viral copies hence higher viral titres. The student t-test analysis on the mean TCID$_{50}$/mL viral titres obtained from the primary and transduced cells gave a P- value of 0.4557, because the P-value was greater than 0.05, this therefore indicated that the variation of the log TCID$_{50}$ /mL when primary cells and the transduced cells were inoculated with dilutions of virus was deemed not significantly different. The TCID$_{50}$/mL results suggest that the transduced cells and primary cells offer similar sensitivities to infection with each virus.

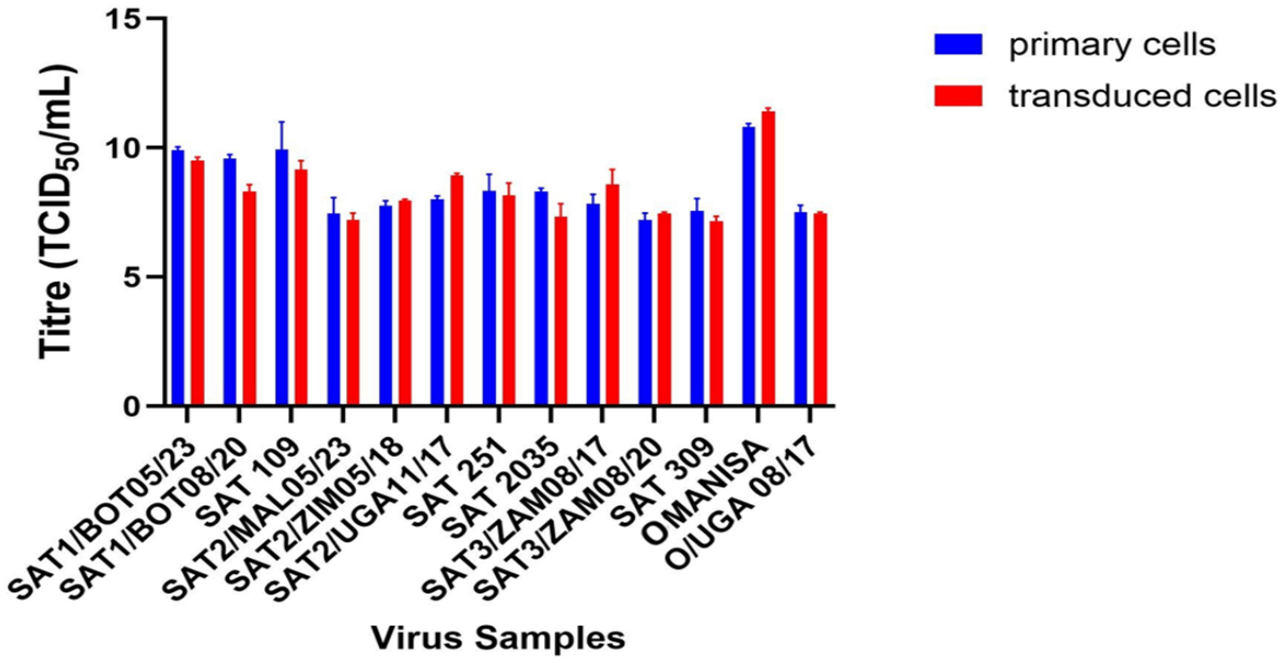


Figure 14: Mean TCID50/mL titres for primary and transduced cells.

Comparison of the sensitivity to infection of primary and transduced cells with vaccine and field strains of FMDV belonging to SAT and O serotypes

4.8 Virus isolation

Viral isolation is crucial for identifying and characterising the virus, as well as for amplifying live virus from original tissue samples for further investigations (Zabal & Fondevila, 2013). Virus isolation confirms the presence of an infectious virus, distinguishing it from non-infectious viral particles. Shorter virus isolation times are desired because it is an indicator of the cells are sensitive to the virus and therefore potentially speeding up diagnostic time. The FMDV serotypes of interest in the current work were the SAT viruses. Their isolation was performed in parallel to FMDV serotype O that has broad geographic prevalence. Virus isolation was performed using passage 15 of the transduced cells and passage 2 of the primary cells. Passage 2 of primary cells is the most commonly used passage for virus isolation and passage 15 of transduced cells had high and consistent growth rates and these passages were targeted for virus isolation. The duration that the virus took to induce CPE in both primary and transduced cells was comparable. CPE was observed on both primary cells and transduced cells indicating that primary and transduced cells were able to effectively isolate FMDV belonging to SAT and O serotypes in 16-24 hours (Figure 15). SAT 1 field strains and the vaccine strain SAT 109 resulted in depiction of CPE in 16 hours.

All the SAT 2 field isolates and vaccine strains were able to induce CPE on both transduced cells and primary cells. All selected SAT 3 samples were able to induce CPE on both the primary cells and the transduced cells within 24 hrs while the vaccine strain SAT 309 took 18 hours to induce CPE. 100% CPE was also observed on both the primary cells and the transduced cells following inoculation with FMDV serotype O samples. O Manisa induced 100% CPE in both cell types in 16 hours, which was faster than O/UGA 08/17 which induced 100% CPE in 24hrs. The time taken to induce 100% CPE was comparable for all the virus samples on the primary cells and the transduced cells. Both the primary and transduced cells were able to isolate both field and vaccine (culture adapted) strains.

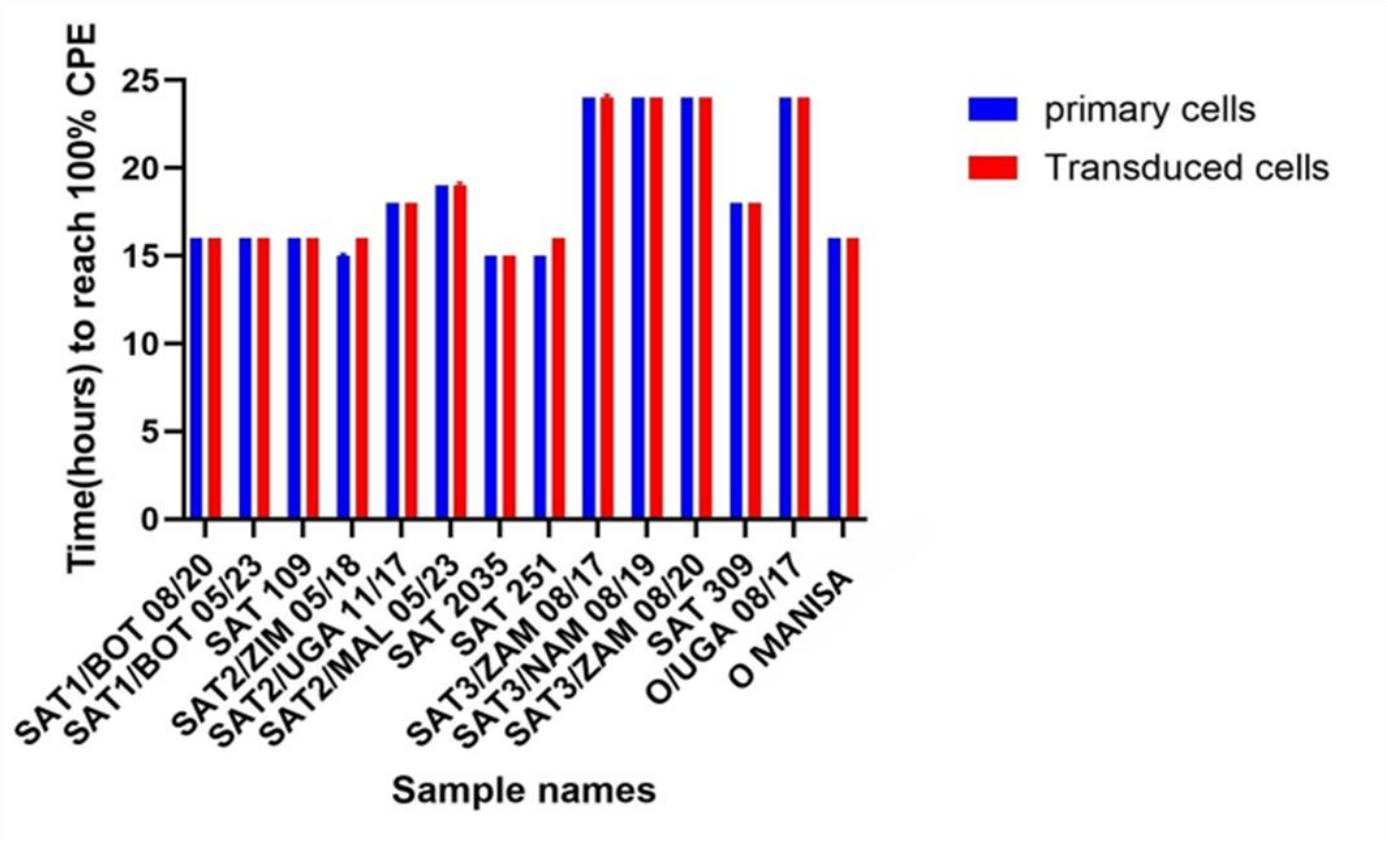


Figure 15: Virus isolation - SAT and O serotypes of FMDV

SAT sand O serotypes of FMDV were cultured in primary and transduced cells. The time taken to induce 100% CPE is reported.

5.0 Discussion

Primary lamb kidney cells were successfully transduced by overexpression of *TERT* delivered into the genome of the cells using lentivirus constructs. The transduced cells survived more passages as compared to primary cells. Similar results were observed by Mao et al., (2018) who were also able to successfully establish a stable bovine thyroid cell line for investigating FMDV through *TERT* over expression. Another cell line that was successfully established by over expression of *TERT* is the primary ovine foetal turbinate cell line which was established by Sun et al., (2022). Steele et al., (2010) too, were able to successfully immortalise principal cells from mouse collecting duct through *TERT* overexpression. These results indicate that indeed TERT can successfully be over-expressed in cells and increase their longevity. The current study was therefore able to accept the hypothesis that primary lamb kidney cells can be immortalised to create a highly sensitive, stable cell line for routine FMDV diagnosis based on the results that shall be discussed herein. The $TCID_{50}$/mL results that were attained when utilising primary and transduced cells were not significantly different for SAT and O serotypes of FMDV, indicating that the transduced cell have comparative performance to primary cells. The transduced cells in this study were passaged up to passage 18 but literature supports extending the number of passages to confirm the stability of morphology, growth patterns, viability, and sustained susceptibility to FMDV infection (Mao et al., 2018; Sun et al., 2022). This is important because banked cells can generally be used up to more than 100 passages, prior to manifestation of any cellular changes that can alter the utility of the cells for their chosen application.

Puromycin concentrations ranging from 1-11 µL/mL were used to assess the selective antibiotic concentration suitable for primary lamb kidney cells. The established concentration of puromycin for selection of primary lamb kidney cells which was applied for selection after transduction was 1 µg/mL in 3 days. Similar results were obtained by Wang et al., (2022) as they found 1 µg/mL as the suitable concentration of puromycin to select yak rumen epithelial cell lines cultured for 96 hours. Miyata et al., (2022) however, reports a selection concentration of puromycin 3 µg/ mL for 3 days for human hepatocytes. In Chinese hamster ovary-K1 (CHO-K1), the puromycin selection concentration was 6 µg/mL (Mohammadkhani et al., 2023). These findings highlight that different cells respond differently to different concentrations of puromycin, therefore determination of a kill curve for every cell type is important. If a kill curve is not properly determined, the original parental cells may survive without having undergone transduction, or the transfected cells may be killed (Delrue et al., 2018). The selective antibiotic

concentration is expected to effectively eliminate non-transduced parental primary cells, while the stably transduced cells will survive selection due to the introduction of puromycin resistance genes (Delrue et al., 2018).

After successfully establishing the kill curve, the primary lamb kidney cells were transduced with *TERT* mediated through lentiviruses. The lentiviruses also introduced puromycin resistance to the lamb kidney cells, enabling them to survive puromycin concentrations which eliminate primary cells. For transduction, primary cells at 80% confluence were exposed to 1µg/mL lentivirus in 5µg/mL of TransduceIT. Cell confluence is an essential factor for achieving successful transduction as insufficient confluency can result in cytotoxicity and reduced transduction efficacy (Storck et al., 2017). The transduction efficiency observed through antibiotic selection was generally low but not atypical at an estimated level of about 3%. Shitova et al., (2024) reports that transduction typically occurs in approximately 5 –10% of cells and that antibiotic-based selection can cause cytotoxicity resulting in the mortality of transduced cells. Kalidasan et al., (2021), also reports a reduced transduction efficiency in human embryonic kidney (HEK293T) cells compared to transfection via non-viral vectors. The efficacy of transduction is contingent upon various parameters, including the purity of nucleic acids, duration of the transfection procedure, type of transfection reagent utilised, and the individual cell lines employed (Jamour et al., 2024). Electroporation is reported to be a better technique in laboratories to achieve enhanced transduction efficiency but overexpressing *TERT* through lentiviruses was preferred as lentiviruses are reported to usually cause less cell morphological alterations in the transduced cells (Jamour et al., 2024; Sun et al., 2022).

Following transduction, the *TERT* expression levels of the transduced and primary cells were measured using RT-qPCR. *TERT* was expressed and detected in the transduced cells in all passages used. This was expected because TERT was over-expressed through lentivirus transduction, therefore its levels would be higher in transduced cells. In primary cells, *TERT* was detected only at passage 3 but could not be detected at passage 5 and 7, which supported the cells' eventual failure to adhere and grow beyond passage 7. A study undertaken by He et al., (2016) indicated that telomerase activity was detected in primary cells before passage 5 and was undetectable by passage 10, which supports the decrease of telomerase levels as cell passages progress in primary cells. *TERT* is reported to decline as telomeres erode with each cell division, and this is reported to be the primary cause of cellular senescence in the majority of somatic cells (Mao et al., 2018). The overexpression of the *TERT* gene and the activation of telomerase are essential mechanisms for achieving cellular immortality. The degree of *TERT* expression is a crucial attribute of

cells immortalised through telomerase reactivation and is expected to rise following immortalisation (Shitova et al., 2024). The $-2^{\Delta\Delta CT}$ results indicated that passage 4 of the transduced cells had a -15,31-fold change in its expression of *TERT*, P10 had a -11,4644-fold change in its expression of TERT, and lastly P18 had a -4,2141- fold change in the expression of *TERT*. This was an indication that the transduced cells were expressing the *TERT* gene more efficiently than the primary cells but the *TERT* levels were reducing as passages progressed. He et al., (2016), report to have isolated *TERT* transcript segments at passages 0, 10, 20, 30, and 40. They also reported that the initial amounts of *TERT* expressed exhibited a reduction by passage 10 and stabilised at a comparable level across passages by passage 40. Sun et al., (2022) however, reports experiencing no significant difference between the *TERT*-expressing ovine kidney cells at passages 15 and 20. It seems that TERT expression levels change until they are stabilised, therefore future iterations of the reported work would extend passaging to at least 30 passages to determine when and what levels *TERT* expressions stabilise.

After confirmation of successful transduction, the transduced and primary cells were allowed to grow in continuous culture. The transduced cells were passaged 18 times. Cell passages can be categorised as early (less than 10 passages), middle (P11–20), and late (more than P20) (Lian et al., 2016). Weiskirchen et al., (2023) reports that there are no specific guidelines regarding the optimal passage range, however, common practice is to not use cells after 30 passages. Early and middle passages are mostly preferred for use in diagnostic assays since they are regarded to be as close as possible to the parental cells (Prasad et al., 2023). The primary kidney cells cultured in this study only lasted up to passage 7 highlighting the shortcoming of using them for routine diagnosis. Seridi et al., (2021) reports that primary cell cultures are useful only during the initial passages, typically within two to four passages since somatic cells have a limited ability to divide and multiply in vitro. Somatic cells have limited *TERT* expression, due to this, telomeric DNA shortens with each cell division until it reaches a critical point of erosion following a predetermined number of cellular replications. The cell machinery interprets this as severe DNA damage, which triggers replicative senescence and apoptosis (Altamura et al., 2020; Sun et al., 2022). He et al., (2016) supports the above finding by reporting that their porcine fibroblast cell line maintained active proliferation until passage 40, while the parent cells exhibited senescence at passage 10. These findings were further confirmed by Mao et al., (2018) who reported a decline in the proliferation of the primary BTY cells post passage 6 while a cell line developed from BTY cells exhibited a consistent growth rate from passages 5-200. These findings indicate that cell transduced with *TERT* have the potential to be

continuously expanded without limitations. Banking such cells has utility for routine diagnosis because they can be retrieved from storage for extended utility and consistency in diagnostic assays.

The morphology of the transduced lamb kidney cells at the beginning of cell culture was monitored until passage 18. This was important to keep track of any morphological changes that the transduced cells might undergo due to lentiviral transduction. It is important to monitor any phenotypical alterations that the cell line may experience as this might indicate a divergence from their initial characteristics. Morphologically altered cell lines cannot accurately represent the cells from whence they originated (He et al., 2016). The transduced cells in the reported work were able to maintain confluent layers with the characteristic shape of primary cells, from passages 4 to 18. A population of spindle shaped and rounded cells was observed in both primary cells and transduced cells, with the tendency of rounded cells increasing in transduced cells as the passages increased. Porter et al., (1973) reports that cell cycle dependent morphology can be experienced in a population of cells as the cells anticipate mitosis. Cells that are about to undergo mitosis are reported to round up in shape. HeLa and Vero cells (green monkey kidney cells) reportedly round up during mitosis as well (Lancaster et al., 2013; Moussa et al., 2020). The appearance of rounded cells was therefore attributed to cell cycle morphology changes. Generally, in comparison to primary cells, no significant morphological alterations were noted in transduced cells under light microscopy. Similar outcomes were also observed by Mao et al., (2018) who reported that the morphology of the cells in their bovine thyroid cell line was similar to that of the primary cells. Wang et al., (2022) also reported that the yak ruminal epithelial primary cells which were transduced with lentivirus exhibited cell morphology that closely resembled that of the primary cells after puromycin screening and cultivation. Evtushenko et al., (2021) did not observe any clear dependence between morphology and immortalization in fibroblasts, thus concluding that immortalization does not have a significant impact on fibroblast morphology. Sun et al., (2022) also reported that in comparison to primary cells, *TERT*-expressing cells exhibited no significant morphological alterations under optical microscopy examination. Cell morphology in the reported work was fundamental for exploring cellular phenotypical behaviour with the progression of cell passages.

Other properties of the primary and transduced cells such as confluency, viability, and cell count per passage were monitored. The transduced cells were able to form desired confluency through all the passages, and the primary cells' confluency began to reduce as they neared ceasing growth at passage 8. Confluent monolayers were also reported by Sun et al., (2022) with progressive passaging of their foetal

turbinate ovine cell line. Handl et al., (2020) reports that human proximal tubular HK-2 cells were able to proliferate to confluent monolayers, and retain the primary HK-2 cells' fundamental morphological and functional characteristics. The ability of the cells to grow and reach confluency is a very important aspect to track in continuous culture as the cell density has to be increased for *in vitro* applications. In terms of cell viability monitoring, viability was initially higher in primary cells compared to transduced cells, but the viability of the transduced cells eventually increased as the passages progressed. Similarly, the cell viability assay of an ovine kidney cell line established by Sun et al., (2022) also revealed that *TERT*-expressing cells from passages 15 to 20 proliferated more rapidly than the corresponding primary cells. Their results support the finding that viability of transduced cells increases with passaging. Viability is an important aspect for cell culture. At the beginning of every passage, it is critical to assess if the cells are still viable. The transduction process affected and slowed down the growth of transduced cells initially (passage 4). This was not an anomalous observation since transduction, trypsinization, and storage can affect viability (Ammerman et al., 2008). The cell count of the cells was another aspect that was monitored. The cell counts of the primary cells regressed with progressive passaging as those of transduced cells increased. Omeir et al., (2015) reports that in the initial 30 passages post-initiation of their canine kidney cell line (CKB1-3T7), cells exhibited a progressive enhancement in cell number, which supports the gradual increase of cell number experienced in this study. Wang et al., (2022) report observations of increased rate of cell adhesion, accompanied by an enhancement in both proliferation speed and vitality with successive passaging. Their findings indicated that immortalised cell lines can undergo continuous proliferation and exhibit high cell viability and growth with continuous passaging.

Relative sensitivity of primary cells and transduced cells to infection with SAT and O serotypes of FMDV was assessed using $TCID_{50}$/mL. The viral titres obtained from primary and transduced by quantification through the $TCID_{50}$/mL assay were compared. The $TCID_{50}$/mL titre results indicated that there was no significant variation in the sensitivity of both the primary and transduced cells to infection with field and vaccine strains belonging to SAT and O serotypes of FMDV as their $TCID_{50}$/mL values were nearly identical. Thus, it was determined that each SAT and O serotypes of FMDV tested were capable of infecting both the cell line and the primary cells with no significant difference. A foetal turbinate ovine cell line which was overexpressed with *TERT* was reported to retain similar levels of viral titres when compared to their primary cells (Sun et al., 2022). Sun et al., (2022), further reported no significant variance of their infectious dose results between their primary cells and their established cells line, as their $TCID_{50}$/mL values were nearly identical. The sensitivity results obtained were also consistent with those

of Zabal & Fondevila, (2013) who obtained favourable results for their ovine kidney cell line (OVK) which was sensitive to FMDV serotypes A and C. Their analysis and results indicated that the OVK cell line was suitable for FMDV-sensitive detection since it portrayed superior performance to BHK 21. Matsuura et al., (2011) and Sun et al., (2022) also created ovine kidney cell lines, and in both studies the ability of both cell lines to retain sensitivity to viral infections after transfection was highlighted. The ability of transduced cells to be comparable to the primary cells offers a much better advantage to tackle batch to batch variation problems exhibited by primary cells. Cell lines offer greater consistency due to homogeneous genotypic and phenotypic features (Mao et al., 2018). The use of cells bearing homogeneous traits is important to avoid differences in assay performance that can be caused by cells derived from different animals. These data suggest that transduced cells can be used for diagnostic assays and produce similar outcomes as primary cells.

Virus isolation was determined by observation of CPE in primary and transduced cell cultures. Positive samples which were observed through visualisation of CPE, were realised at the same culture times for primary and transduced cells. The transduced cells and primary cells were able to isolate the field strains and vaccine strains of FMDV and reach 100% CPE within the same time intervals for all SAT and O serotypes of FMDV. Zabal & Fondevila, (2013) states that the ovine kidney cell line that they established was able to successfully isolate FMDV, supporting the finding in this study. The comparison of virus isolation between primary and transduced cells is crucial since primary cells have been previously reported to depict shorter culture times when infected with FMDV as compared to transduced cells (Brehm et al., 2009; Sarry et al., 2024). Conducting virus isolation is essential to establish if the transduced cells can respond to FMDV virus infection in the same time frames as the primary lamb kidney cells as such also determining its suitability for diagnostic and fundamental research purposes. The determination if the of transduced cells is comparable to primary cells is a critical step in establishing a long-lasting alternative to primary lamb kidney cells.

6.0 Conclusions

The general aim of this research was to develop and assess a cell line that is appropriate for propagation of FMDV. Primary lamb kidney cells were successfully immortalised through lentiviral mediated transduction. After transduction, 3% of the initial cells survived antibiotic selection and these were passaged further. Successful transduction was confirmed by comparative determination of the expression levels of TERT in both primary and transduced cells through RT-qPCR. The primary cells ceased proliferation at passage 8 while the transduced cells-maintained growth to passage 18. The transduced cells were able to maintain confluent monolayers, the cell counts and viability of the cells gradually increased until they stabilised from passage 10 to 18. The primary cells and the transduced cells gave comparable results when characterised with $TCID_{50}$ and virus isolation. The virus isolation times were comparable when both field and vaccine strains were inoculated into cultures of both primary and transduced cells. The student-t test results showed that there was no significant difference in the titres of SAT and O serotypes of FMDV produced by primary and transduced cells using $TCID_{50}$/mL assay. The results offer promise for the transduced cells to be further developed and validated towards substitution of primary lamb kidney cells in FMD diagnostic assays. The presented work is one of few intentional efforts to develop a cell line for propagation of SAT serotypes of FMDV. SAT serotypes of FMDV are prevalent in the sub-Saharan region, thus, having a cell culture system that is sensitive to these viruses will aid in having a consistent supply of cells for diagnostic cells and eventually aiding in the control of FMD in the region.

6.1 Limitations and future work

As part of future work, cells will be serial diluted after transduction and colonies with stable expressions of *TERT* will be selected for further passaging and validation of diagnostic tests. This will potentially address reducing levels of *TERT* expression encountered in this study. As another way of addressing the declining levels of TERT expression, the reproducibility of the RT-qPCR data will also be conducted. Future research with additional time and money will focus on reproducibility, which is an important component of cell line development. A cell line's development frequently entails dynamic changes in gene expression; if the results cannot be replicated, it is challenging to determine with certainty whether the upregulation is the product of biological processes or merely experimental variance. Gene upregulation or lack thereof will be guaranteed to be a genuine biological event through reproducibility rather than a chance mistake brought on by technological problems like inaccurate pipetting, RNA degradation, or variations in reverse transcription efficiency (Diaba-Nuhoho & Amponsah-Offeh, 2021).

Passaging will also be extended to determine the number of passages that the transduced cells can be passaged before reaching a notable reduction in performance. Further passaging is crucial to evaluate the transduced cells' capacity to sustain their growth characteristics and determine if there will be any observations of genetic or phenotypic drift. Extended passaging of the transduced cell will also aid in allowing the *TERT* expression levels to be extensively studied. This will allow insight into whether *TERT* expressions are still consistently expressed across passages as well as confirm the efficiency of the lentiviral transduction. As part of future work, the cells will be passaged using media that will include fungicide together with antibiotics. This will be important since the continuous passaging of the cells was limited by product contamination. The lack of fungicide in the media had a major limitation in the number of passages that the cells underwent in the study. The passages beyond 18 were not included as part of the results as these cells were mostly contaminated by fungal species. The study was conducted in a controlled environment but there was a problem of high humidity, which supported the growth of moulds and yeast.

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Appendix 1

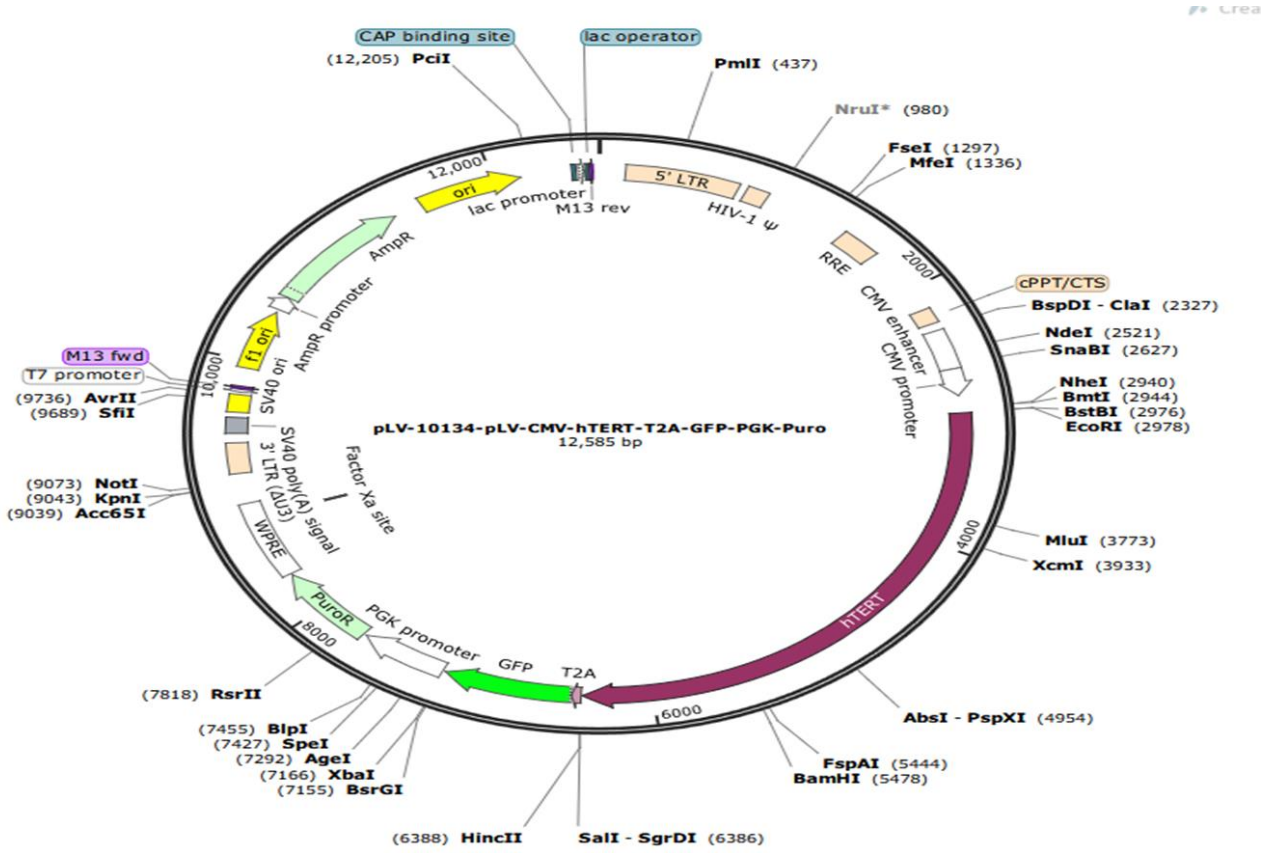


Figure 16: hTERT lentivirus construct (https://www.cellomicstech.com/Images/pLV-10134-pLV-CMV-hTERT-T2A-GFP-PGK-Puro%20Map.pdf)

Table 2: RNA quantification and quality

Sample name	A260/280	A260/230
Transduced cells (P4)	2.05	2.02
Transduced cells (P10)	1.95	1.84
Transduced cells (P18)	2.03	1.82
Primary cells (P3)	2.02	2.27
Primary cells (P5)	2.01	1.89
Primary cells (P7)	1.97	1.86