

2011

## Developing a Quantitative PCR Assay for Detecting Viral Vector Shedding from Animals

Swathee Chinnasamy  
*Wright State University*

Follow this and additional works at: [https://corescholar.libraries.wright.edu/etd\\_all](https://corescholar.libraries.wright.edu/etd_all)



Part of the [Immunology and Infectious Disease Commons](#), and the [Microbiology Commons](#)

---

### Repository Citation

Chinnasamy, Swathee, "Developing a Quantitative PCR Assay for Detecting Viral Vector Shedding from Animals" (2011). *Browse all Theses and Dissertations*. 1073.  
[https://corescholar.libraries.wright.edu/etd\\_all/1073](https://corescholar.libraries.wright.edu/etd_all/1073)

This Thesis is brought to you for free and open access by the Theses and Dissertations at CORE Scholar. It has been accepted for inclusion in Browse all Theses and Dissertations by an authorized administrator of CORE Scholar. For more information, please contact [library-corescholar@wright.edu](mailto:library-corescholar@wright.edu).

**DEVELOPING A QUANTITATIVE PCR ASSAY FOR  
DETECTING VIRAL VECTOR SHEDDING FROM  
ANIMALS**

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

By

SWATHEE CHINNASAMY

M.Sc BIOTECHNOLOGY, BHARATHIAR UNIVERSITY, 2009

2011

WRIGHT STATE UNIVERSITY

WRIGHT STATE UNIVERSITY

SCHOOL OF GRADUATE STUDIES

DECEMBER 13, 2011

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Swathee Chinnasamy ENTITLED Developing a quantitative PCR assay for detecting viral vector shedding from animals BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

---

Dawn P. Wooley, Ph.D.  
Thesis Director

---

Barbara E. Hull, Ph.D.  
Program Director

Committee on Final Examination

---

Dawn P. Wooley, Ph.D.

---

Adrian Corbett, Ph.D.

---

Katherine Excoffon, Ph.D.

---

Andrew Hsu,  
Dean, School of Graduate Studies

## **ABSTRACT**

Chinnasamy, Swathee. M.S., Microbiology and Immunology Program, Wright State University, 2011. Developing a quantitative PCR assay for detecting viral vector shedding from animals.

Replication deficient viruses have been used widely for replacing, repairing, and deleting target genes. These recombinant viruses are tested on research animals or patients in clinical trials. Although viral vectors distribute in the body, they are also disseminated into the environment through secretion and excretion processes. By studying the extent of shedding, a proper risk assessment can be performed and appropriate biocontainment can be achieved.

Adenoviral and lentiviral vectors were produced from commercially available kits. The transgene present in both vector systems was the *lacZ* reporter gene encoding for  $\beta$ -galactosidase. Primers and probes were designed for the encapsidation region of the adenoviral vector and the Rev Response Element (RRE) of the lentiviral vector to

detect the viral vectors by Q-PCR. Internal control sequences were also designed and synthesized for both systems. Standard curves were generated using five 10-fold serial dilutions. Singleplex reactions confirmed the specificity of the probes on the viral DNA and the quality control sequences. A comparison of singleplex with multiplex data was performed to validate the assay. The assay was used to quantify the total virus genome count (vg) present in adenoviral and lentiviral stocks. The vg counts were compared to the infectious titers in order to gauge the quality of the virus stocks. For the lentivirus stock, a second confirmatory assay was made by p24 assay using enzyme linked immunosorbent assay to validate the Q-PCR results. The efficiency of cDNA synthesis (to convert the lentiviral RNA into cDNA for Q-PCR) was determined to be 20% on average.

Overall, a highly specific and sensitive Q-PCR assay was developed that will enable researchers to quantify the amount of adenoviral and lentiviral vector shedding from infected animal hosts. This assay will help to determine proper housing requirements for research animals and improve worker safety.

## TABLE OF CONTENTS

	PAGE
<b>I. INTRODUCTION</b> .....	1
Adenovirus as a vector.....	2
Lentivirus as a vector.....	6
Biodistribution of adenoviral and lentiviral vectors	8
Shedding <i>via</i> excretion.....	10
<b>II. MATERIALS AND METHODS</b> .....	12
Vector Production.....	12
Adenoviral primers and probe.....	17
Lentiviral primers and probe.....	21
Quality control (QC) sequences.....	25
RNA production and isolation.....	27
RT-PCR.....	28
Quantitative PCR (Q-PCR).....	28
p24 assay.....	29
<b>III. RESULTS</b> .....	30
Successful construction of adenoviral vector.....	30
Successful construction of lentiviral vector.....	31
Assay design.....	33

Generation of standards.....	37
Assay specificity.....	37
Sensitivity of the assay.....	38
Validation of the assay.....	38
Standard graphs for adenoviral vector.....	39
Standard graphs for lentiviral vector.....	43
Efficiency for Reverse Transcription-PCR.....	47
Singleplex test on the recombinant adenovirus and lentivirus.....	50
p24 assay.....	50
<b>IV. DISCUSSION</b> .....	<b>51</b>
<b>V. GLOSSARY</b> .....	<b>58</b>
<b>VI. REFERENCES</b> .....	<b>59</b>

## LIST OF FIGURES

	PAGE
1. An outline of the steps involved in the Ad Easy™ XL Adenoviral vector system.....	14
2. An outline of the steps involved in the pLenti 6.3/V-5 DEST kit.....	16
3. Sequence of Wild Type adenoviral encapsidation region.....	19
4. Sequence of Adenoviral vector Synthetic Fragment (Quality Control).....	20
5. Sequence of Wild Type HIV RRE region.....	23
6. Sequence of HIV Synthetic Fragment (Quality Control).....	24
7. Quality control sequence inserted into plasmids by IDT.....	26
8. Agarose gel analysis of quality control sequence DNA after restriction.....	35
9. Agarose gel analysis of RNA control.....	36
10. Standard curve of Multiplex Adenoviral vector.....	40
11. Standard curve of Adenovirus Wild-type Sequence...	41
12. Standard curve of Adenovirus quality control	



	sequence.....	42
13.	Standard calibration curve of Multiplex Lentiviral vector.....	44
14.	Standard calibration curve of Lentiviral vector Wild-type Sequence.....	45
15.	Standard curve of Lentivirus QC Sequence.....	46
16.	Standard curve of Lentiviral vector control sequence template-RNA.....	48
17.	Comparison of the DNA copy numbers obtained from RNA after reverse transcription-PCR.....	49

## LIST OF TABLES

	PAGE
1. Sequence information of primers/probes used for adenoviral vector.....	18
2. Sequence information of primers/probes used for lentiviral vector.....	22

## **ACKNOWLEDGEMENTS**

I would like to sincerely thank Dr. Dawn P. Wooley for her constant guidance, support and encouragement throughout my project. I am fortunate to have an advisor from whom I learnt perfection, patience, hard work and to be organized in the laboratory. I thank my committee members Dr. Adrian Corbett and Dr. Katherine Excoffon for their support, patience and advice. I would also like to thank the program director, Dr. Barbara Hull for her valuable advice throughout the program. Lastly, I would like to thank my parents, my brother and my friends—Shashi, Anusha and Madhuri—for their love, support and encouragement which kept me going.

## II. INTRODUCTION:

Gene therapy is a potential form of treatment for a variety of acquired and inherited diseases. The therapy involves introducing genetic material into target cells to slow a disease process or cure a particular condition. The treatment is accomplished by using gene transfer vectors that can litteringl different cells, organs, or tissues.

Vectors can be engineered to possess a variety of desirable characteristics, such as having the ability to be produced in large quantity and concentrated form, to litteringl different cell types (both dividing and non-dividing cells), to be stably expressed (either by integration into host DNA or by formation of an episome), and to avoid unwanted immune responses.

Gene delivery may be classified into two categories: non-viral and viral. The non-viral gene delivery methods include chemical transfection, lipofection, or electroporation of naked DNA. Although naked DNA vectors have the advantage of being produced in high concentration and eliciting less immune response, they suffer from transient gene expression and inefficient gene transfer.

Viral vectors provide a more efficient way of transferring a desired gene into target cells since they have naturally evolved to deliver their own RNA or DNA genomes into host cells (Verma & Weitzman, 2005). In general, viruses are capable of inserting their genetic material into host cells for replication and have evolved over time to escape host immune responses. One strategy for gene therapy is to replace some of the viral genes with the desired foreign genes (transgenes) and to engineer the virus to target a desired target cell population. Some viruses that are commonly used as vectors include adenovirus, lentivirus, retrovirus, adeno-associated virus, and herpes simplex virus (Warnock, Daigre, & Al-Rubeai, 2011).

**Adenovirus as a vector:**

Adenoviruses represent the largest non-enveloped viruses, having achieved the largest size that can be transported via endosomes. The virus structure is comprised of an icosahedral capsid and double-stranded DNA genome of about 26-45 kb (Volpers & Kochanek, 2004). Adenovirus was originally isolated in 1953 from adenoid tissue. Since then, more than 50 serotypes have been discovered and also classified into six species A-F (Warnock et al., 2011).

They cause upper respiratory diseases, bladder infections, and gastroenteritis, depending on the serotype.

Adenovirus capsids have a pentameric penton base protein from which the trimeric fiber protein extends into a globular knob domain. The virus attaches to its cellular receptor using the globular knob domain of the fiber protein (Volpers & Kochanek, 2004). Depending on the serotype, adenoviruses use a variety of cellular receptors, including the Coxsackie-Adenovirus receptor, other immunoglobulin superfamily proteins, integrins, bitter sulfate glycosaminoglycans, cluster of differentiation antigens, sialic acid, and other proteins and non-protein molecules (Zhang & Bergelson, 2005). The arginylglycylaspartic acid tripeptide (RGD) motif in the penton base protein interacts with surface integrin molecules of the cell, leading to internalization of the virus by means of clathrin-dependent receptor mediated endocytosis. Following the internalization of the virus, the capsid is dismantled in the cytoplasm and the core protein-coated viral genome is transported in to the nucleus (Russell, 2009).

The Adenovirus genome consists of two regions for transcription—the early and the late regions. The early region contains four transcription units. The first gene

transcribed is E1A, which codes for a transactivator protein for the other early region genes, E1B, E2, E3 and E4. The E2 gene codes for DNA polymerase, preterminal protein, and DNA binding protein, which are required for viral replication. The E3 region encodes for proteins that have anti-immune functions. The E4 region encodes for proteins involved in mRNA transport and splicing (Warnock *et al.*, 2011). Adenovirus type 5 (Ad5) is the most commonly used serotype for vector design. First generation vectors have the E1 and E3 genes deleted, thus preventing the virus from replicating and providing space for foreign gene insertion. Since the E1 gene is essential for replication, the vectors must be complemented. This is generally accomplished by using the human embryonic kidney cell line named 293, which expresses E1 proteins (Bett *et al.*, 1994). This system allows for high titers to be produced, but the lack of E3 causes the virus to be cleared from the system due to the host immune response, thus leading to short duration of transgene expression. A major limitation of adenoviral vectors it is that they induce inflammation, are neutralized by host antibodies, and exhibit short term expression of transgenes (Kovesdi *et al.*, 1997).

Two approaches have been traditionally used to generate recombinant adenovirus vectors. The first method

has been to directly ligate the gene of interest into the adenoviral genome. This method turned challenging as the virus is large and there were not many unique restriction sites. Another approach was developed where the gene of interest is cloned into a shuttle vector followed by transferring the gene into the virus genome via homologous recombination in mammalian packaging cell lines. The disadvantage here is that defective adenovirus was produced, and the screening and purification of plaques and repeated purification was laborious (Mittal et al. 1993; Stratford-Perricaudet, et al. 1992).

Later, a method to reduce the time consumption for producing a recombinant adenovirus was introduced that utilized the homologous recombination carried out in prokaryotic cells (bacteria), giving a better selection system (He et al., 1998). The recombinant adenovirus is produced by a double recombination process between a plasmid vector, which has an adenoviral backbone, and a shuttle vector, which carries the gene of interest. To avoid the background caused by non-recombinant shuttle plasmid, the bacteria are pre-transformed with the plasmid. The recombinant adenovirus is selected by resistance to antibiotic. The recombinant adenovirus is then packaged in mammalian cell lines which complement with the E1 gene *in*



*trans* that is required for replication. This system has been commercialized by Agilent Technologies (formerly Stratagene) as the AdEasy™ system.

### **Lentivirus as a vector:**

Although retroviruses in the *Gammaretrovirus* genus of the *Retroviridae* family had been used widely for delivering genes into target cells, they had the disadvantage of not being able to infect the non-dividing cells. When it was discovered that viruses in the *Lentivirus* genus of the *Retroviridae* family contained nuclear localization signals on the Matrix, Vpr, and Integrase proteins that allow them to cross the nuclear membrane in the absence of mitosis, interest grew in developing lentiviral vectors for gene delivery.

One of the most commonly studied lentiviruses is HIV-1. Some of the other lentiviruses include *Bovine immunodeficiency virus* (BIV), *Simian immunodeficiency virus* (SIV) and *Feline immunodeficiency virus* (FIV). The genome organization of HIV-1 is similar to that of other retroviruses, but it has additional regulatory and accessory genes that lead to a more complex replication cycle. Its RNA genome is about 10 kb and its capsid is

about 100 nm in diameter (Volpers *et al.*, 2004). The HIV-1 regulatory proteins are Tat and Rev. The former helps the viral RNA to be produced by activating the viral promoter. The Rev protein promotes the transport of unspliced RNA from the nucleus to the cytoplasm by interacting with the Rev responsive element (RRE) present on the viral RNA.

In general, retroviruses enter cells by fusion with the plasma membrane. The exact mechanism of uncoating is unclear. Reverse transcription takes place in the cytoplasm, and the double-stranded DNA intermediate is transported to the nucleus where it integrates into the host chromosome. At this point, the virus is under control of the cellular transcription machinery.

Many of the early HIV vectors were almost intact viral genomes having deletions in the *env* gene, which was provided *in trans*. Use of the wild-type HIV Env limited the target cell population to CD4<sup>+</sup> cells. Replacing the HIV Env with other viral envelope proteins (such as VSV-G, MLV amphotropic envelope glycoprotein) resulted in a wider target of cell types and higher titer values.

The chances of producing a replication competent virus was reduced by producing the second and third generation vectors and also the self inactivating

lentivirus vector (SIN) (Dull et al., 1998; Miyoshi et al., 1998; Zufferey et al., 1998). Separate plasmids were used in all these vector systems for envelope genes and packaging genes, but the third generation system uses a fourth plasmid encoding the rev gene in addition to the above features. The third generation vectors had the Tat dependent promoter replaced with *Rous sarcoma virus* (RSV) promoter. Thus, the most widely used lentiviral vectors are the third generation vectors since they reduce the sequence homology with the wild type HIV (Dull et al., 1998).

#### **Biodistribution of Adenoviral and Lentiviral vectors:**

Therapeutic effect is achieved on a pathological process by means of gene transfer to a target tissue. Possible expression of the transgene in non-target tissue is a drawback for gene therapy. One of the main characteristic features to be studied for a vector used in gene therapy is biodistribution in tissues (Pan et al., 2002). Biodistribution study aims at defining areas where the vector spreads, target organs for toxicity, and germline transmission risk assessment. Some of the commonly studied organs are heart, brain, liver, spleen, kidney, lungs. One of the most widely used methods to detect

toxicity based on genetic material transferred is quantitative PCR (Q-PCR). Prior to clinical trial, these studies are generally conducted on cell lines and in animal models (Hiltunen et al., 2000).

Adenoviral vector biodistribution has been studied previously, and it was found to be predominantly expressed in the liver when injected into mice through intravenous route (IV, tail vein injection) (Alemany, et al. 2000; Wood et al., 1999). The IV route also resulted in expression in most organs, including the lung, kidney, heart, and spleen. A study involving the distribution of the human-multi drug resistance gene (MDR1) was evaluated using the mice colon carcinoma model, in which the adenovirus was administered by bone marrow transplantation. By means of *in situ* hybridization and immunohistochemistry, they demonstrated that the vector was expressed in the lung, kidney, intestine, and bone marrow cells, and not in the liver, spleen, brain, or tumor (Zhao et al., 2010).

Hiltunen et al. (2000) showed that biodistribution depended on the route of administration. They administered adenovirus vector virus to rabbits through periadventitial and intravascular routes and showed that both methods lead to systemic leakage of virus and expression in non-target organs such as the bone marrow, liver, skeletal muscle, and

testis. Less leakage was observed with the periadventitial delivery route. Biodistribution is followed by shedding and the data for both are required for analyzing the safety profile (Tiesjema, et al. 2010).

### **Shedding via Excretion:**

The dissemination of viral vector into the environment by means of excreta is referred as shedding. It is considered a major risk factor to the environment and therefore shedding analysis of urine and feces are performed. Again, shedding depends on the route of administration since it leads to biodistribution to those organs from where it sheds. Three possible routes have been studied for shedding via urine and they are through blood and kidney, from bladder and from prostrate through the ureter. Biodistribution of the virus to the liver and secretion of bile remains as a possible route for shedding via feces. Kawahira *et al* (2009), in their analysis of adenoviral shedding after esophagus cancer treatment, showed that 29.7% percentage of patients were positive for shedding via feces and also 10% patients showed positive for shedding *via* urine, suggesting that gastrointestinal can be a possible route for shedding. Treatment of lung cancer

with adenoviral vector also showed positive stool samples (Griscelli et al., 2003). The I.V route leading to biodistribution to kidney acts as a possible route for adenoviral vector shedding through urine and other routes include intra-prostatic and intramuscular. (Tiesjema et al., 2010).

Quantification of the shedding is performed using Q-PCR. Some of the other methods include conventional PCR and transgene expression. In case of patients treated with viral vectors the potential for transmission to third parties needs to be considered if shedding is observed. Other factors to be considered are that shedding is time and dose dependent. One of the most important parameters for performing successful Q-PCR is the use of a control, which is sometimes spiked into the actual test sample, for checking inhibition, specificity, and sensitivity of the reaction. Reactions for Q-PCR are often performed in duplicate or triplicate to increase accuracy (Gonin & Gaillard, 2004). The technique of using an internal control in the same reaction tube (multiplexing) offers the advantage of greater accuracy, but this control can also compete against the amplification, if not chosen carefully and validated.

### III. MATERIALS AND METHODS:

#### Vector production:

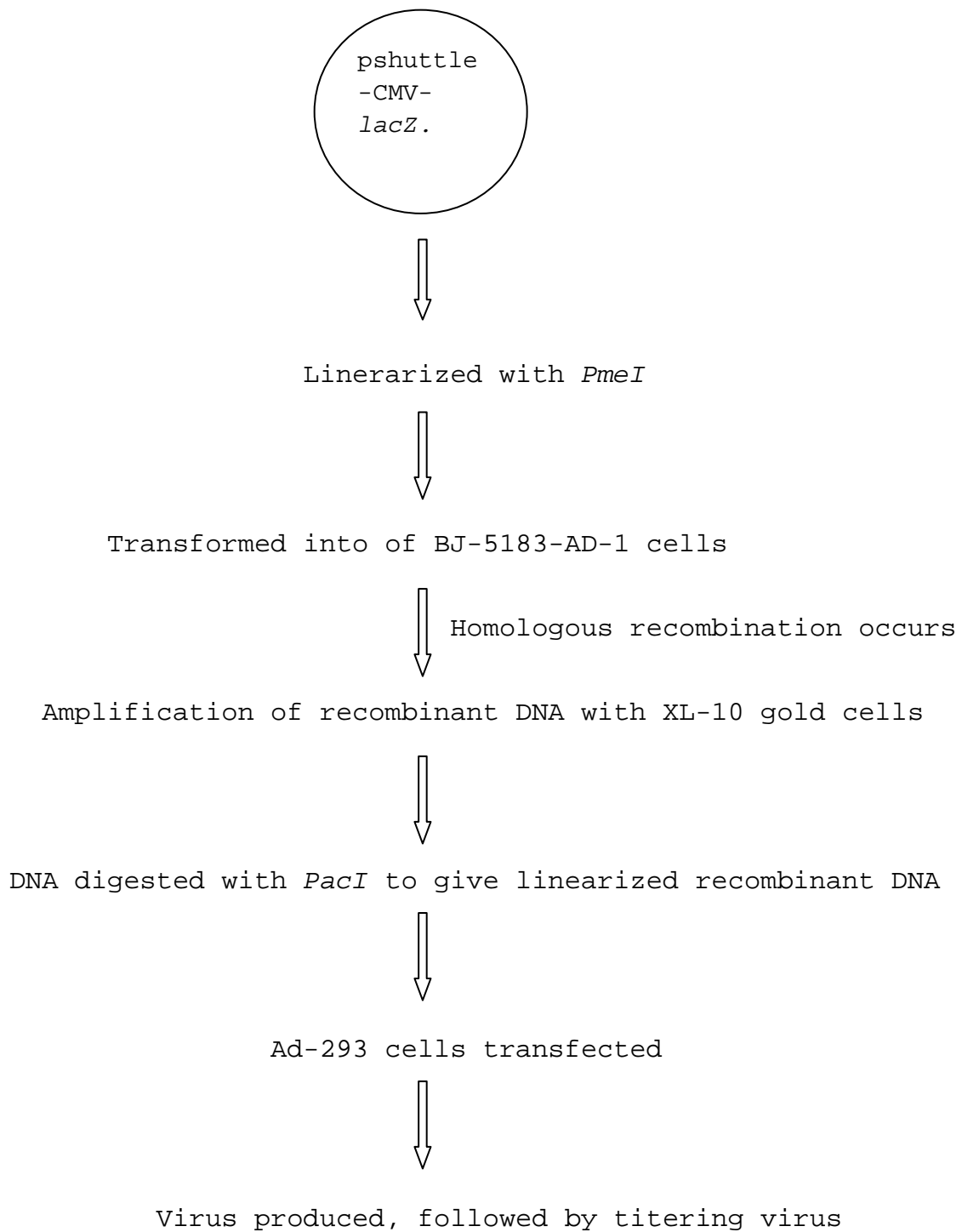
##### Adenoviral vector:

The Ad Easy™ XL Adenoviral vector system (Agilent Technologies, Santa clara, CA) was used for the production of adenoviral vector using the manufacturer's instruction. The following is a brief description of the protocol.

The pshuttle-CMV-lacZ vector encoding the gene for  $\beta$ -galactosidase was linearized with the enzyme PmeI (New England Biolabs, Ipswich, MA) and transformed into BJ5183-AD-1 competent cells and plated. The smallest colonies were chosen. DNA was isolated, checked for the desired size, and transformed into the XL-10 Gold cells for amplification of the plasmid. Individual colonies from the transformation reaction were selected and restricted with PacI (New England Biolabs) and analyzed for the correct restriction pattern. A maxi prep (QIAGEN, Valencia, CA) was performed to isolate a large amount of plasmid DNA from a large culture, and it was stored at 4°C. Plasmid DNA for transfection was restricted PacI (New England Biolabs, Ipswich, MA) and purified using the StrataPrep PCR Purification Kit (Agilent Technologies). The AD-293 cell

line was established and passaged as per the manufacturer's instruction. This cell line was used for transfection of the recombinant Ad5 plasmid to produce recombinant adenovirus. The primary viral stocks were produced, stored at -70°C, and titered using a plaque assay. A second round of infection was performed on the cells to amplify the viral stock which was then purified and concentrated on a binding column using the AdEasy Purification kit (Agilent Technologies). The stocks were titered again using plaque assay.

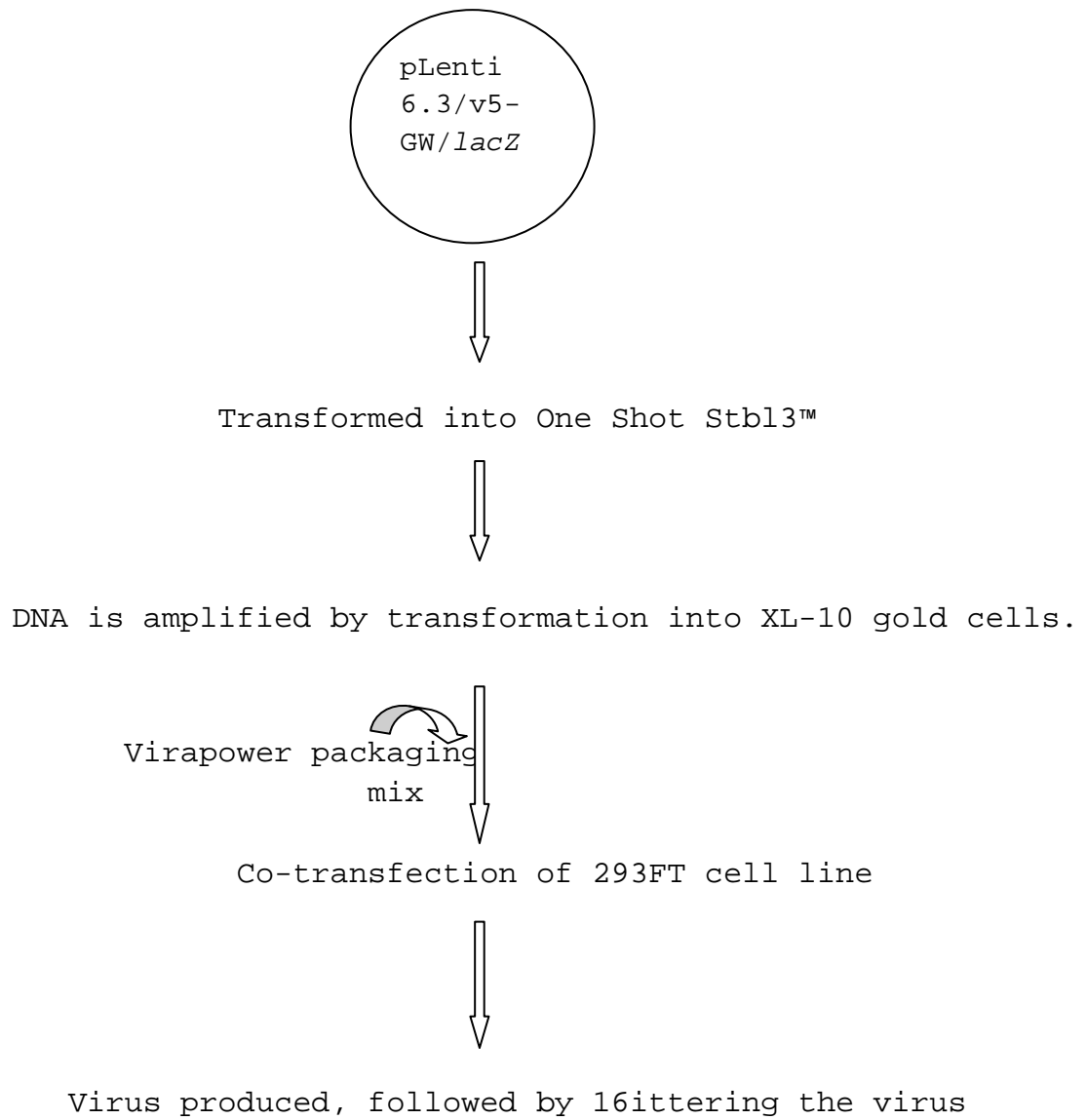




**Fig 1: An outline of the steps involved in the Ad Easy™ XL Adenoviral vector system.**

Lentiviral vector:

The lentiviral (HIV) vector was produced using pLenti 6.3/V-5 DEST kit (Invitrogen, Carlsbad, CA). The vector containing the *lacZ* gene with pLenti-DEST was transformed into One Shot Stbl3™ cells that were provided with the kit and plated. Colonies were selected and cultured for plasmid isolation using a mini prep kit (QIAGEN). Restriction with XhoI (New England Biolabs) enzyme was performed to confirm the presence of the correct DNA size. This DNA was used to transform XL-10 gold cells, selected on agar plates containing kanamycin, and cultured. Plasmid DNA was isolated by maxi prep (QIAGEN). It was restricted with XhoI (New England Biolabs) to confirm the restriction pattern. The restricted DNA was used to transfect the 293FT cell line along with the ViraPower Lentiviral Expression Systems (Invitrogen) to produce recombinant lentiviral vector virus.



**Fig 2: An outline of the steps involved in the pLenti 6.3/V-5 DEST kit.**

**Adenoviral primers and probes:**

The wild type probe for the adenoviral vector was labeled at the 5' end with reporter dye molecule 6-carboxyfluorescein (6-FAM) whose emission wavelength is 520 nm and at the 3' end with Black Hole Quencher-1 with a quenching wavelength between 520 and 583 nm. The QC probe for the adenoviral vector was labeled at the 5' end with reporter dye molecule 6-carboxyfluorescein-4',5'-dichloro-2',7'-dimethoxyfluorescein (JOE) whose emission wavelength is 555 nm and at the 3' end with Black Hole Quencher-2 with a quenching wavelength between 550 and 668 nm. For detection of the encapsidation region which starts at 183 bp in the adenoviral vector, the primers were designed using MacVector software to yield a final amplicon of 116 bp.

**TABLE 1: SEQUENCE INFORMATION OF PRIMERS/PROBES USED FOR ADENOVIRAL VECTOR.**

<b>PRIMER/PROBE</b>	<b>SEQUENCE</b>	<b>NUMBER OF BASEPAIRS</b>	<b>MELTING TEMPERATURE</b>
FORWARD PRIMER	5'-ACA GGA AGT GAC AAT TTT CGC-3'	21	66.0°C
REVERSE PRIMER	5'-TCA GAT TTC ACT TCC TCT TAT TCA G-3'	25	64.69°C
PAdeASY WT PROBE	5'-/56-FAM/ACT CGG TTA CGC CCA AAT TTA CTA CAA CAT/3BHQ -1/-3'	30	71.62°C
CONTROL SEQUENCE PROBE	5'-/56-JOEN/ ACT ATG CCA CGC TCA CAT GTA TTA CAT CGT/3BHQ-1/-3'	30	72.65°C

Encapsidation Start

166	176	186	196	206	216	226
*	*	*	*	*	*	*

GATGTGGCAAAAGTGACGTTTTTGGTGTGCGCCGGTGTACACAGGAAGTGACAATTTTCGCGCGGTTTTA  
CTACACCGTTTTCACTGCAAAAACCACACGCGGCCACATGTGTCTTCACTGTTAAAAGCGCGCCAAAAT

236	246	256	266	276	286	296
*	*	*	*	*	*	*

GGCGGATGTTGTAGTAAATTTGGGCGTAACCGAGTAAGATTTGGCCATTTTCGCGGGAAAACCTGAATAAG  
CCGCCTACAACATCATTTAAACCGCATTGGCTCATTTCTAAACCGTAAAAGCGCCCTTTTGACTTATTC

306	316	326	336
*	*	*	*

AGGAAGTGAATCTGAATAATTTTGTGTTACTCATAGCGCGTAA  
TCCTTCACTTTAGACTTATTA AAAACACAATGAGTATCGCGCATT

Encapsidation End

**Fig 3: Sequence of Wild Type adenoviral encapsidation region.**

The sequence of the encapsidation region on the adenoviral vector for which the primers and probe were designed. Sequences in red : forward and reverse primers and the probe sequences are double *underlined*.

Encapsidation Start

166	176	186	196	206	216	226
*	*	*	*	*	*	*

GATGTGGCAAAAGTGACGTTTTTGGTGTGCGCCGGTGTACACAGGAAGTGACAATTTTCGCGCGGTTTTA  
CTACACCGTTTTCACTGCAAAAACCACACGCGGCCACATGTGTCTTCACTGTTAAAAGCGCGCCAAAAT

236	246	256	266	276	286	296
*	*	*	*	*	*	*

GGCGGACGATGTAATACATGTGAGCGT**GGC**ATAGTAAGATTTGGCCATTTTCGCGGGAAAACCTGAATAAG  
CCGCCTGCTACATTATGTACACTCGCACCGTATCATTCTAAACCGTAAAAGCGCCCTTTTGACTTATTC

306	316	326	336
*	*	*	*

AGGAAGTGAAATCTGAATAATTTTGTGTTACTCATAGCGCGTAA  
TCCTTCACTTTAGACTTATTAAAACACAATGAGTATCGCGCATT

Encapsidation End

**Fig 4: Sequence of Adenoviral vector Synthetic Fragment (Quality Control).**

The sequences in red indicate the forward and reverse primers; the probe sequences are double *underlined* and the mutated base pairs are indicated in **BOLD**. This entire region was inserted into pIDTSMART (Integrated DNA technologies, San Jose, CA).

**Lentiviral primers and probes:**

Similar to the adenovirus system, the probe for the wild type lentiviral vector was labeled at the 5' end with 6-FAM and at the 3' end BHQ-1, whereas the probe for the lentiviral QC sequence was labeled at the 5' end with JOE and at the 3' end with BHQ-2. The region for detection was the Rev Responsive Element (RRE) starting at 1075 bp of the lentiviral vector. For detection of the HIV RRE region, which starts at 1075 bp in the HIV vector, the primers were designed using MacVector software to yield a final amplicon of 111 bp.



**TABLE 2: SEQUENCE INFORMATION OF PRIMERS/PROBES USED FOR LENTIVIRAL VECTOR.**

<b>PRIMER/PROBE</b>	<b>SEQUENCE</b>	<b>NUMBER OF BASEPAIRS</b>	<b>MELTING TEMPERATURE</b>
FORWARD PRIMER	5'-TCA ATG ACG CTG ACG GTA C-3'	19	65.28°C
REVERSE PRIMER	5' /GAC TGT GAG TTG CAA CAG ATG/3'	21	63.88°C
p Lenti RRE WT PROBE	5'/56- FAM/ TGT TCT GCT GCT GCA CTA TAC CAG ACA/3BHQ- 2/3'	27	71.84°C
CONTROL SEQUENCE PROBE	5'/56- JOEN/TGC TCT ACC ACT GAA CTC TAC GAG TCT/3BHQ-2/3'	27	72.26°C

```

RRE Start
|
|   1084   1094   1104   1114   1124   1134   1144
|   *     *     *     *     *     *     *
AGGAGCTTTGTTCCCTTGGGTTCTTGGGAGCAGCAGGAAGCACTATGGGCGCAGCGTCAATGACGCTGACG
TCCTCGAAACAAGGAACCCAAGAACCCTCGTCGTCCTTCGTGATACCCGCGTCGCAGTTACTGCGACTGC

   1154   1164   1174   1184   1194   1204   1214
   *     *     *     *     *     *     *
GTACAGGCCAGACAATTATTGTCTGGTATAGTGCAGCAGCAGAACAATTTGCTGAGGGGCTATTGAGGCGC
CATGTCCGGTCTGTTAATAACAGACCATATCACGTCGTCGTCTTGTTAAACGACTCCCGATAACTCCGCG

   1224   1234   1244   1254   1264   1274   1284
   *     *     *     *     *     *     *
AACAGCATCTGTTGCAACTCACAGTCTGGGGCATCAAGCAGCTCCAGGCAAGAATCCTGGCTGTGGAAAG
TTGTCGTAGACAACGTTGAGTGTTCAGACCCCGTAGTTCGTGAGGTCCGTTCTTAGGACCGACACCTTTC

                                RRE End
   1294   1304   |
   *     *     |
ATACCTAAAGGATCAACAGCTCCT
TATGGATTTCCCTAGTTGTCGAGGA

```

**Fig 5: Sequence of Wild Type HIV RRE region.** The sequence of the RRE region on the lentiviral vector for which the primers and probe were designed. Sequences in red : forward and reverse primers and the probe sequences are double underlined.

```

RRE Start
|
|      1084      1094      1104      1114      1124      1134      1144
|      *        *        *        *        *        *        *
AGGAGCTTTGTTCCCTTGGGTTCTTGGGAGCAGCAGGAAGCACTATGGGCGCAGCGTCAATGACGCTGACG
TCCTCGAAACAAGGAACCCAAGAACCCTCGTCGTCCTTCGTGATACCCGCGTCGCAGTTACTGCGACTGC

      1154      1164      1174      1184      1194      1204      1214
      *        *        *        *        *        *        *
GTACAGGCCAGACAATTATAGACTCGTAGAGTTCAGTGGTAGAGCAATTTGCTGAGGGCTATTGAGGCGC
CATGTCCGGTCTGTTAATATCTGAGCATCTCAAGTCACCATCTCGTTAAACGACTCCCGATAACTCCGCG

      1224      1234      1244      1254      1264      1274      1284
      *        *        *        *        *        *        *
AACAGCATCTGTTGCAACTCACAGTCTGGGGCATCAAGCAGCTCCAGGCAAGAATCCTGGCTGTGGAAAG
TTGTCGTAGACAACGTTGAGTGTGAGACCCCGTAGTTCGTGAGGTCCGTTCTTAGGACCGACACCTTTC

                                RRE End
      1294      1304      |
      *        *        |
ATACCTAAAGGATCAACAGCTCCT
TATGGATTTCTAGTTGTCGAGGA

```

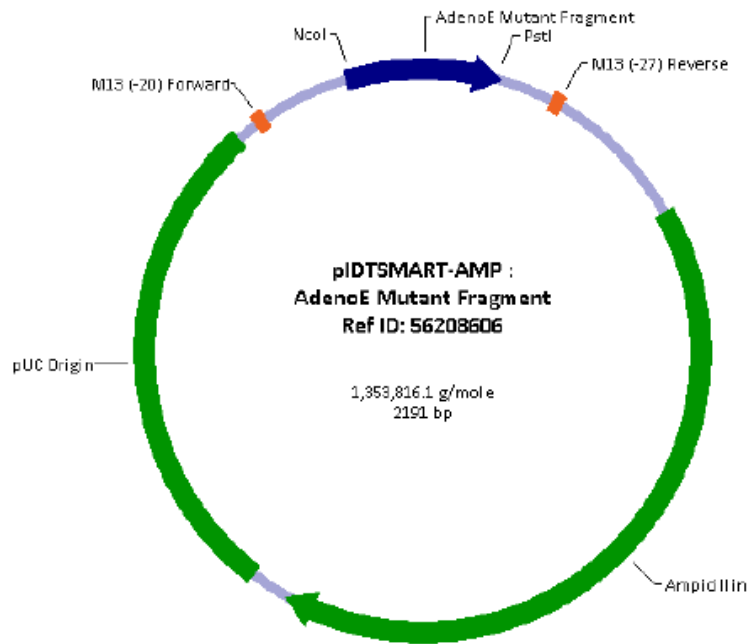
**Fig 6: Sequence of HIV Synthetic Fragment (Quality Control).**

The sequences in red: forward and reverse primers; the probe sequences are double *underlined* and the mutated base pairs are indicated in **BOLD**. This entire region was inserted into pIDTBBLUE (Integrated DNA Technologies).

**Quality control (QC) sequences:**

Quality control sequences were designed for both adenoviral and lentiviral vectors by incorporating specific mutations into the probe region of the synthetic molecule to allow differential detection in PCR. Restriction sites NcoI and PstI for adenoviral and XhoI and NotI for lentiviral control sequence were engineered at the ends of the synthetic molecules for cloning. The adenoviral QC sequence was cloned into the pIDTSMART vector, while the lentiviral QC sequence was cloned into pIDTBlue vector (Integrated DNA Technologies), the latter being an *in vitro* transcription vector.

A)



B)

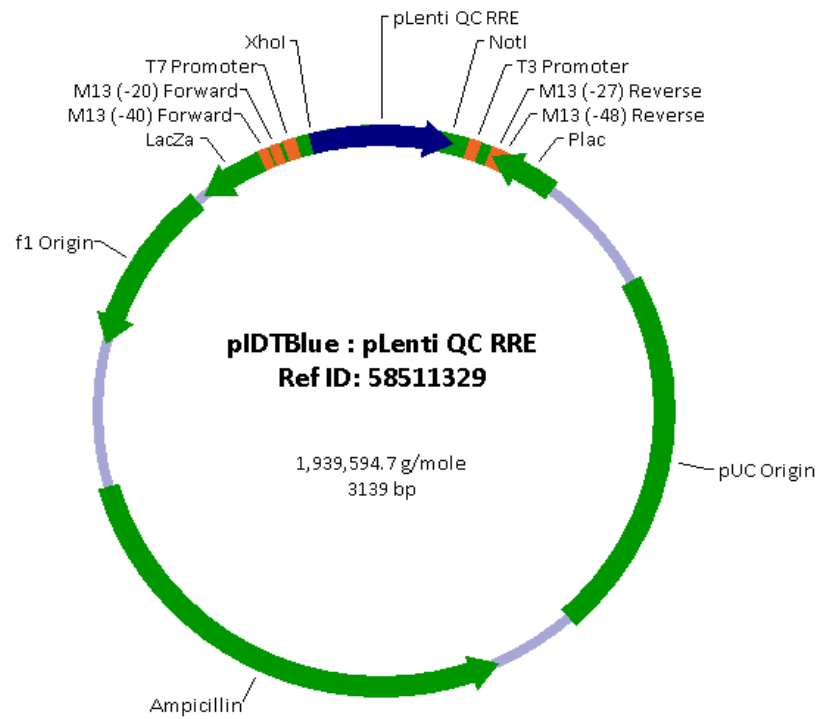


Fig 7: Quality control sequence inserted into plasmids by IDT.

Region in Blue is the QC sequence flanked by restriction sites A) QC plasmid for adenoviral vector; B) QC plasmid for lentiviral vector.

### **RNA production and isolation:**

The lentiviral QC sequence was *in vitro* transcribed using the RiboMAX Large Scale RNA Production System (Promega, Madison, WI). The pIDTBlue vector DNA containing the lentiviral QC sequence was extracted with a phenol:chloroform mixture to enhance cutting with NotI (New England Biolabs). The restricted DNA was separated from uncut DNA by overnight gel electrophoresis using constant amperage of 30 mA, 1.5% agarose gel, and Tris-boric acid-EDTA buffer. The desired linearized plasmid of 3,139 bp was excised from the gel and purified using QIAquick extraction kit (QIAGEN). The *in vitro* transcription reaction was performed using the gel purified plasmid DNA, T7 polymerase, and other RiboMAX reagents at 37°C for four hours. To remove input DNA, RNase-free Dnase was added at a concentration of one unit per microgram of input DNA and incubated at 37°C for 15 minutes. The RNA was purified using SV RNA Isolation System (Promega) following the manufacturer's instructions. The purified RNA was analyzed

for size, purity, and concentration by electrophoresis on a 1% native agarose gel using Tris-acetic acid, EDTA buffer. The RNA was further quantified by UV spectrometry, aliquoted, and stored at -70°C.

#### **RT-PCR:**

The RNA was converted to cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA) in accordance with manufacturer's instructions. Serial 10-fold dilutions of the RNA were made such that there was a range of 1 to  $10^6$  molecules per cDNA reaction. The reaction conditions were 10 minutes at 25°C, 120 minutes at 37°C, 5 minutes at 85°C and  $\infty$  at 4°C. This cDNA was used in Q-PCR to determine the reverse transcription efficiency.

#### **Quantitative PCR (Q-PCR):**

For quantitative PCR analysis of adenovirus and lentivirus genomes, primers and probes were synthesized by IDT (Integrated DNA Technologies). The reactions were performed on the ABI Prism 7900 HT sequence detection system under the following reaction conditions: 1 cycle at

50°C for 2 min, 1 cycle at 95°C for 10 min, and 40 cycles at 95°C for 15 sec and 60°C for 60 sec. A set of DNA standards were produced that corresponded to  $10^6$ ,  $10^5$ ,  $10^4$ ,  $10^3$  and  $10^2$  molecules per Q-PCR reaction. The standards were created by diluting the wild type and quality control plasmid DNAs of the Adenovirus and HIV-1 vectors. The dilution buffer was 10 mM Tris, 1 mM EDTA, pH 7.5. The final Q-PCR reaction volume was 20  $\mu$ l and contained the proprietary TaqMan mix (Applied Biosystems, Carlsbad, CA), which provided all of the necessary reagents for amplification. Primers were used at concentrations of 18  $\mu$ M and probes were used at concentrations of 2.5  $\mu$ M. The graphs and statistical analyses for the standard curves were plotted using SigmaPlot 11.2 software (Systat Software, Inc., San Jose, CA).

**p24 Assay:**

The p24 assay was performed using Retrotek HIV-1 p24 Antigen ELISA kit (Zeptometrix Corporation, Buffalo, NY) by following the manufacturer's protocol.



### III.RESULTS

#### Successful construction of Adenoviral vector:

##### Generation of recombinant Ad plasmid:

The Ad Easy™ XL Adenoviral vector system (Agilent Technologies) makes use of the first generation adenoviral vector system. The two sets of early region genes, E1 and E3, are deleted in this system. The E1 region is required for replication of virus. However, it is provided *in trans* by the AD-293 cells. The recombinant adenoviral plasmid was constructed by transforming the BJ5183-AD-1 competent cells with linearized shuttle vector pshuttle-CMV-LacZ and pAd Easy vector-1. Both the shuttle vector and pAd Easy-1 have the right arm and the left arm regions that provide sequence homology for the process of homologous recombination. The transformed cells were plated and the smallest colonies were chosen and cultured. The recombination was confirmed by isolating the plasmid DNA, restricting with *PacI* (New England Biolabs) and loading onto an agarose gel to visualize a 3.0 kb band showing the recombination occurred between the left and right arms. This DNA was used for amplification of the plasmid using XL-10 gold cells and following a similar selection process, the 3.0 kb band on the gel was observed.

### Viral Vector Production:

Adenovirus was produced by transfection of AD-293 cells with plasmid DNA. After transfection of cells with linearized plasmid DNA, virus was harvested from cells after 8 days for a primary viral stock. The titer of the viral stock was determined by plaque assay. Cells were infected with serial 10-fold dilutions of virus stock in duplicate and overlaid with agarose. Visible plaques appeared after about one week, and they were counted on day 20 post-infection. Using this primary stock, one more round of infection was performed to achieve an increase in viral titer. For this amplification, the virus was harvested after 24-48 hr. The viral stocks were then purified and concentrated by the AdEasy Purification kit (Agilent Technologies). To determine the titer of the stock, a plaque assay was again performed. Two adenovirus stocks were made and had titers of  $6 \times 10^7$  and  $4 \times 10^8$  pfu/ml.

### **Successful construction of Lentiviral Vector:**

The HiPerform™ Lentiviral Expression System (Invitrogen) was used and represents a third generation lentiviral vector system. It comprises four plasmids—one

vector and three helper plasmids. This reduces the chance of producing replication competent virus. The regulatory and accessory genes of HIV coding for as Tat, Rev, Vif, Vpr, Vpu, and Nef are deleted from the vector plasmid, and the third helper plasmid contains VSV-G (*Vesicular stomatitis virus* envelope protein) replacing the *env* gene (Dull et al., 1998). The vector, which expresses the *lacZ* gene encoding for  $\beta$ -galactosidase, was transformed into One shot Stbl3™ bacteria, selected, and cultured. DNA was isolated and restricted with *AflIII* and *XhoI* to confirm integrity. This plasmid was used along with ViraPower™ Packaging Mixture (Invitrogen) to transfect 293FT cells to produce virus. The expression system supplies replication and structural proteins for producing the viral vector. Viral stock was obtained and its titer was determined by using HT1080 cells. These cells were transduced with 10-fold serial dilutions and placed under blasticidin selection 48 hours post-transduction. They were stained with crystal violet after 10 days, and the stained colonies were counted. The final viral stock had a concentration of  $1 \times 10^7$  pfu/ml.

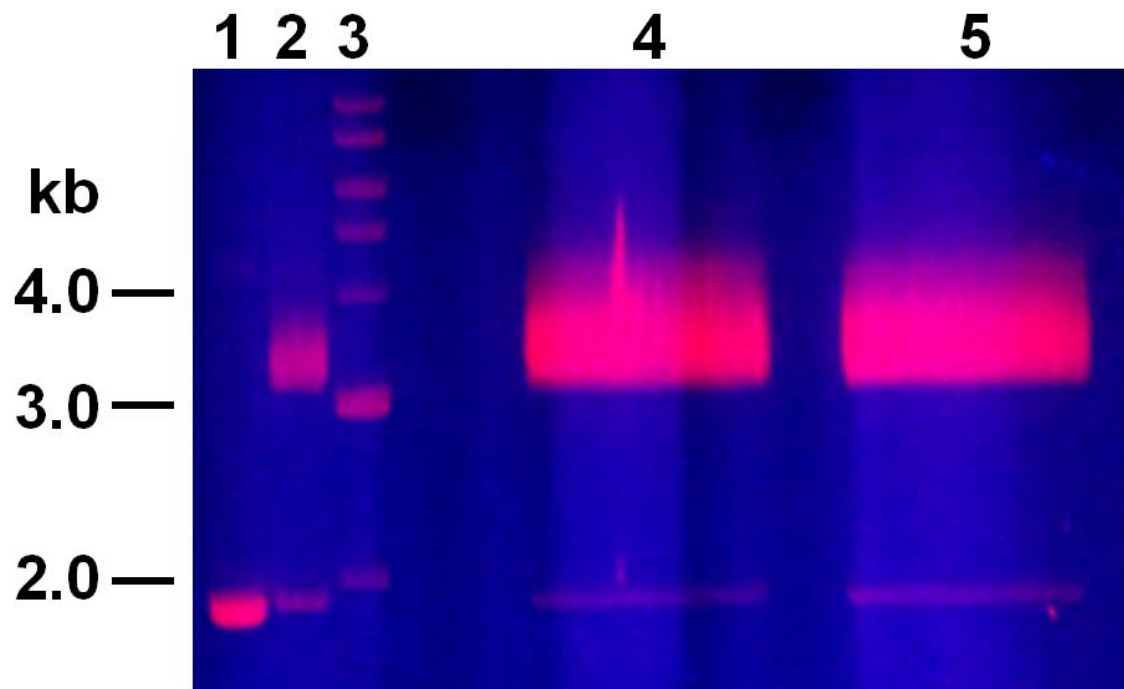
**Assay Design:**

Primers and probes were designed from the DNA sequence of the adenoviral and lentiviral vectors. These primers and probes were designed in a manner that they can be used universally for the current generation of vectors. For adenoviral vectors, they were designed for the encapsidation region. For the lentiviral vector, primers and probes were designed in the Rev Response Element (RRE) region. An internal control was also designed for both the vectors.

Generation of quality control sequence templates:

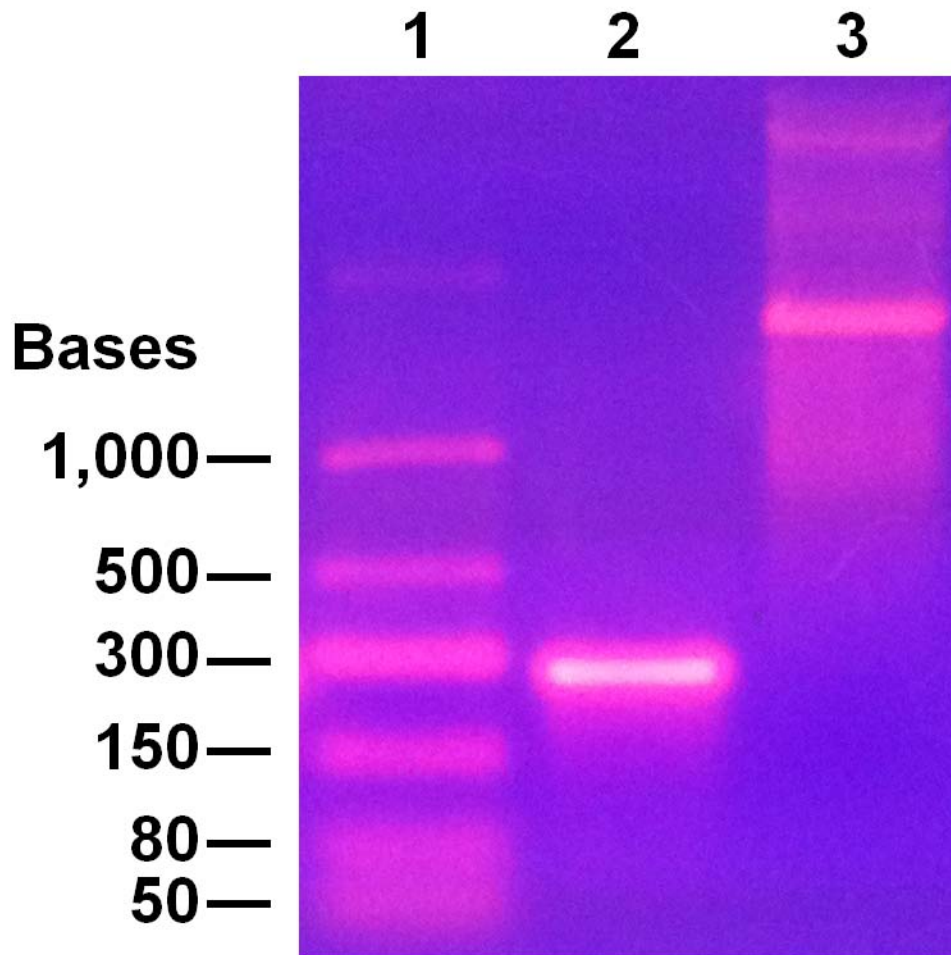
Quality control sequences were designed such that the region of probe binding in the encapsidation region of adenoviral vector had 10 sequence differences; the RRE region of lentiviral vector had 8 differences. They were further designed such that the melting temperatures were nearly identical. These sequences were cloned into two different plasmids for both vectors. These plasmids were transformed into bacteria for large scale production. The plasmids were serially diluted for the use as standards alongside the wild type standards for quantification purposes.

The quality control sequence DNA of lentiviral vector was converted to RNA, since the genetic material of lentiviral vector is RNA. For the conversion, the DNA was extracted with phenol:chloroform for efficient restriction. It was restricted by *Not I* to create a 3' end to terminate transcription. Insufficient cutting was observed when the plasmid DNA by itself was restricted using *Not I* and separated on an agarose gel. Therefore, the plasmid DNA was extracted twice using phenol:chloroform and then restricted. This resulted in more efficient cutting of the DNA. The restricted DNA was loaded onto an agarose gel (Fig 8). The desired band was purified and transcribed into RNA by using RiboMAX™ Large Scale RNA Production System (Promega). The RNA was purified using the SV RNA isolation system (Promega), analyzed by agarose gel electrophoresis, and quantified by spectrophotometry. A single RNA species of 296 bases was produced (Fig. 9). The RNA was diluted to a concentration of  $1 \times 10^{11}$  molecules/ $\mu$ l, aliquoted, and stored at  $-70^{\circ}\text{C}$  for later use as a control.



**Fig 8: Agarose gel analysis of lentiviral quality control sequence DNA after restriction.**

Quality control sequence DNA of lentiviral vector restricted with *Not I* after it was extracted twice by phenol:chloroform. Lane 1: Uncut pLenti RRE QC DNA; Lanes 2,4,5: Restricted pLenti RRE QC DNA showing 3.1 kb band; Lane 3: 1kb DNA Ladder.



**Fig 9: Agarose gel analysis of purified RNA control.**

Lane 1: Low Range ss RNA Ladder; Lanes 2: Purified QC RNA;

Lane 3: Transcriptional control (1,800 bases).

**Generation of standards:**

Ten-fold serial dilutions (representing  $10^6$  to  $10^2$  molecules per microliter) of vector DNAs and the quality control sequences were amplified by Q-PCR in duplicate. A singleplex reaction was performed in which a single template was amplified using one set of primers/probe. The DNA was amplified, quantified, and compared to a known input number. The copy number of the DNA was equal to the input number and optimized as standards. Slopes between -3.1 and -3.6 with efficiencies of reactions between 90% and 110% and with  $R^2$  values between 0.990 and 1.00 were acceptable.

**Assay specificity:**

The specificity of the probes was checked by amplifying the five serial dilutions of wild type DNA with QC primers/probe and the QC DNA with wild type primers/probe in separate wells on the plate and they were compared to the standards. There was no amplification in either of them, thus confirming their specificity.



**Sensitivity of the assay:**

The reactions were performed in multiplex i.e. the primers/probes were for both the wild-type and the control sequence plasmids but with only one of the templates (wt or QC) in the same well on the plate. The template was amplified and they were compared to the standards. There was no change in the sensitivity of the assay i.e. the probes did not inhibit each other. These reactions were checked separately for both adenoviral and lentiviral vectors.

Another multiplex reaction was performed: the primers/probes of both the wt and QC sequences were amplified with both wt and QC templates in the same well. Graphs were plotted showing their slopes, Ct values and efficiencies (Fig 10 & Fig 13). The Ct (cycle threshold) is the number of cycles required for the fluorescent signal to cross the established baseline level.

**Validation of the assay:**

The singleplex and multiplex reactions were compared to validate the assay. Ct values for the wild type template of the vector from both singleplex and multiplex

were compared (Fig 11; Fig 14). Similarly, the Ct values of quality control sequence template were also compared (Fig 12; Fig 15). The Ct difference is calculated and a value less than 1 was acceptable.

**Standard graphs for adenoviral vector:**

The graphs indicate that the primers/probes for both the wild-type and control sequence are highly specific and the templates were optimized as standards.

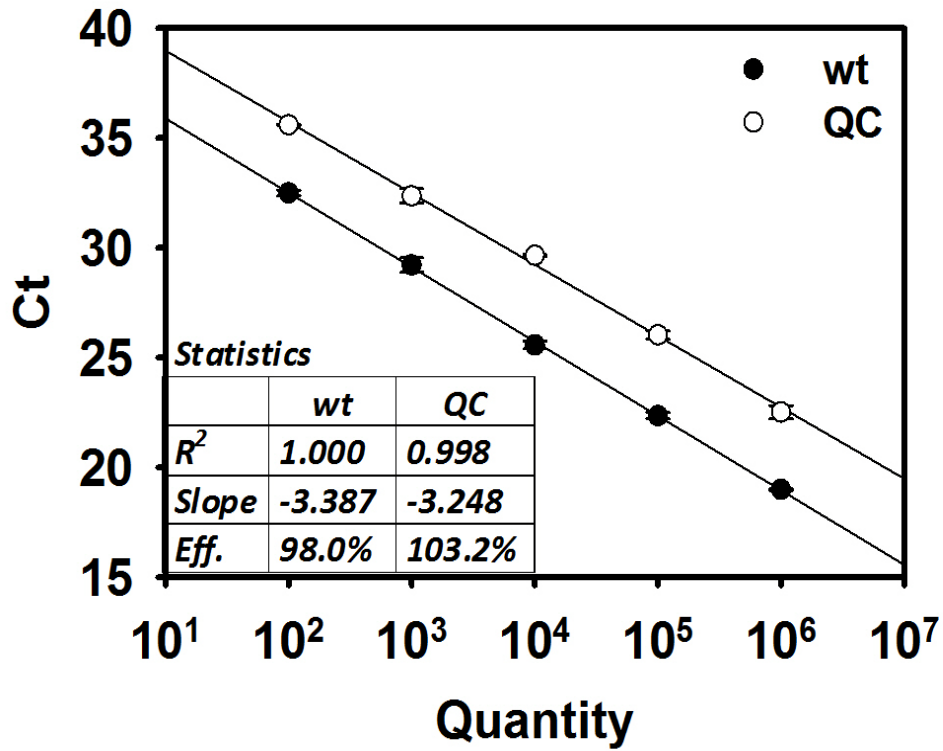
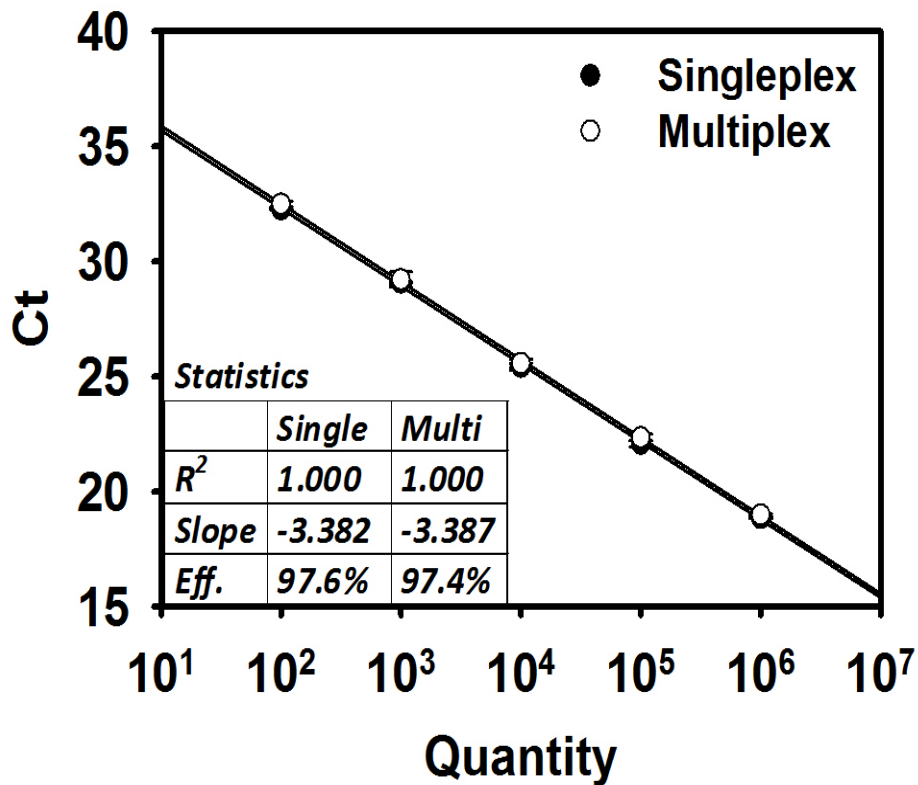


Fig 10: Standard calibration curve of multiplex adenoviral vector.

Quantitative PCR analysis for the adenoviral wild-type and control sequence (multiplex). Five 10-fold serial dilutions were used for the curve and the x-axis of the graph is the Ct values against the copy numbers on the y-axis. The Ct difference between them was less than one. The values of  $R^2$ , efficiency and slope fall within the standard required number.



**Fig 11: Standard curve of adenovirus Wild-type Sequence.**

To validate the multiplex assay format, Singleplex Vs Multiplex Ct values were compared for adenoviral vector wild-type sequence which was analyzed on Q-PCR. The efficiency,  $R^2$  and the slope of the wild type in singleplex was the same as in multiplex with their average Ct difference being 0.19.

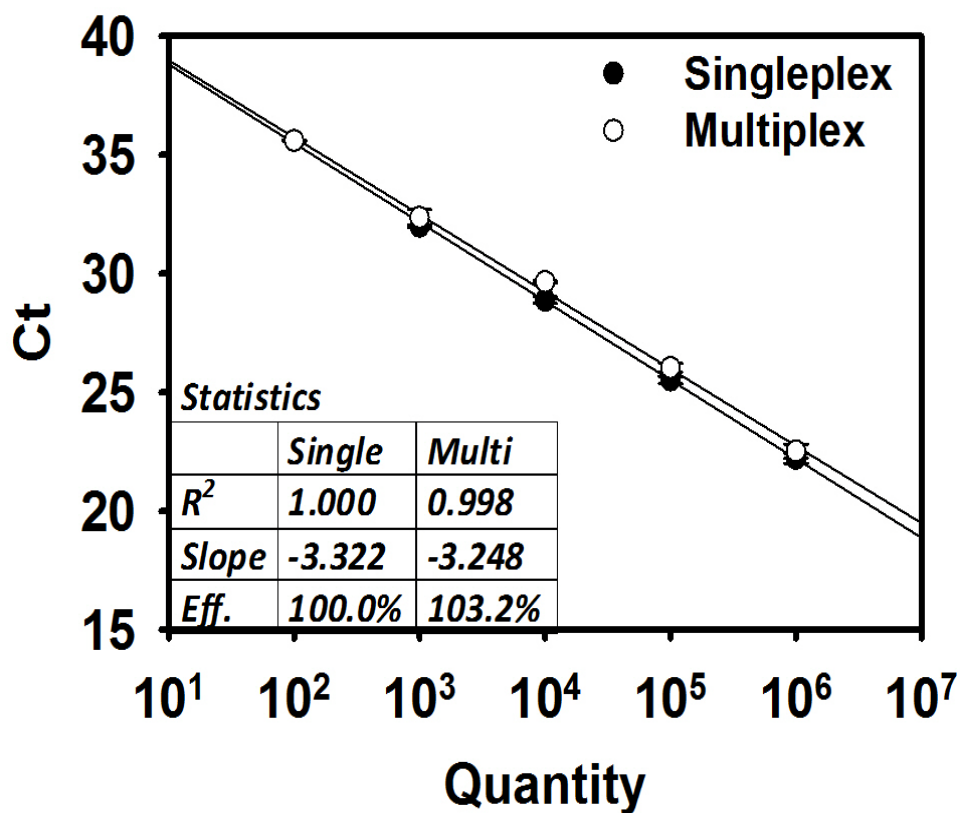
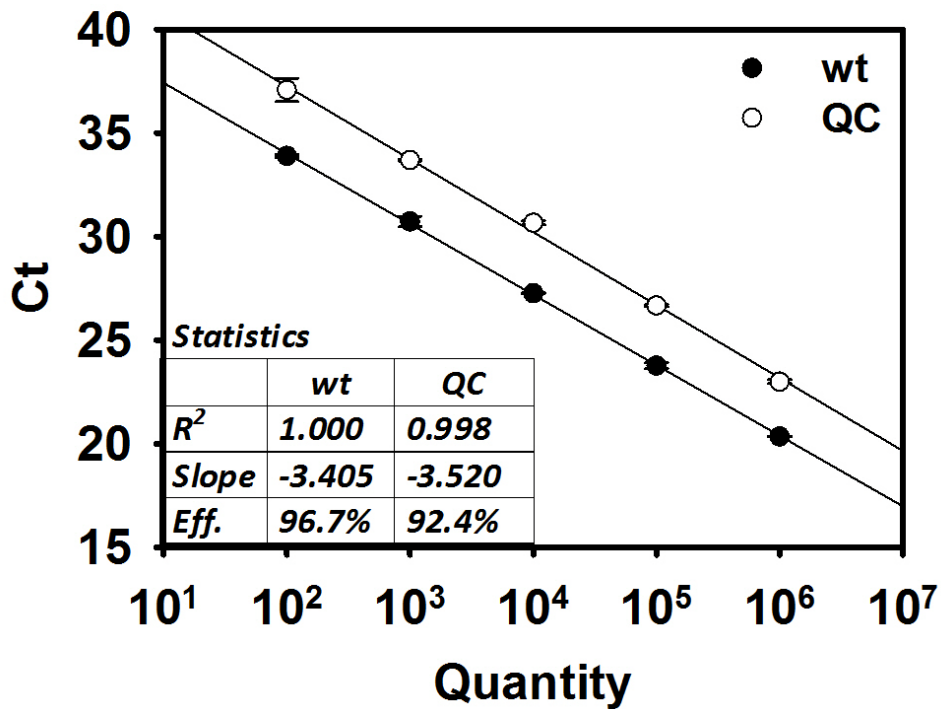


Fig 12: Standard curve of adenovirus quality control sequence.

To validate the multiplex assay format, singleplex Vs Multiplex Ct values were compared for adenoviral vector control sequence plasmid which was analyzed on Q-PCR. The values of  $R^2$ , Efficiency and slope for QC were almost close numbers in both the singleplex and multiplex although the average Ct difference between the two was calculated to be 0.39.

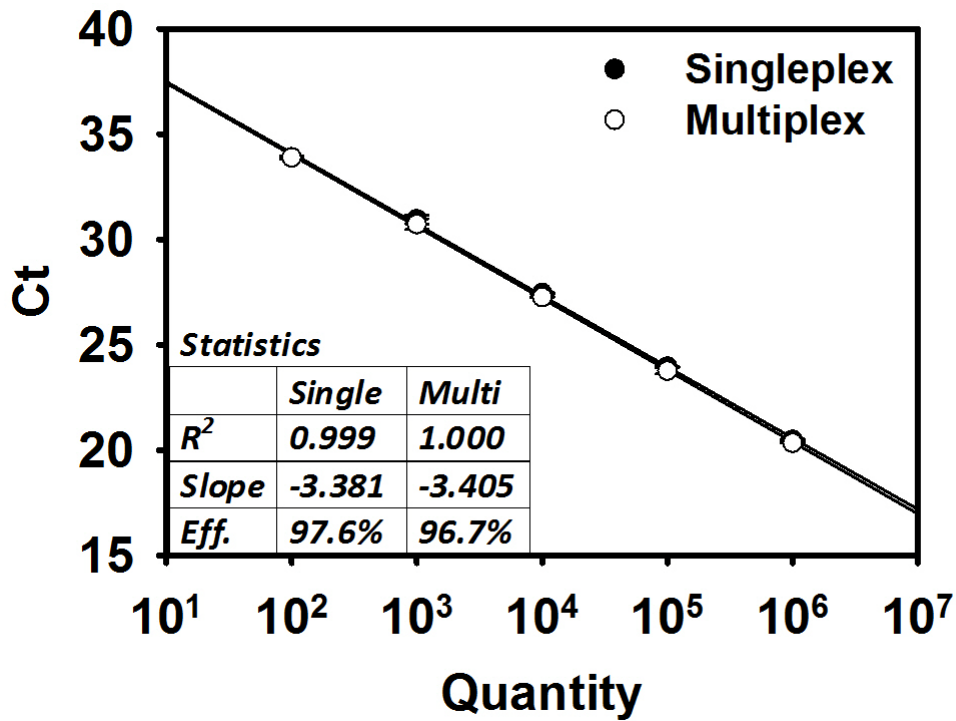
**Standard graphs for lentiviral vector:**

The statistics from the graph indicate high specificity for the primers/probes to the templates of wild-type and control sequence.



**Fig 13: Standard calibration curve of multiplex lentiviral vector.**

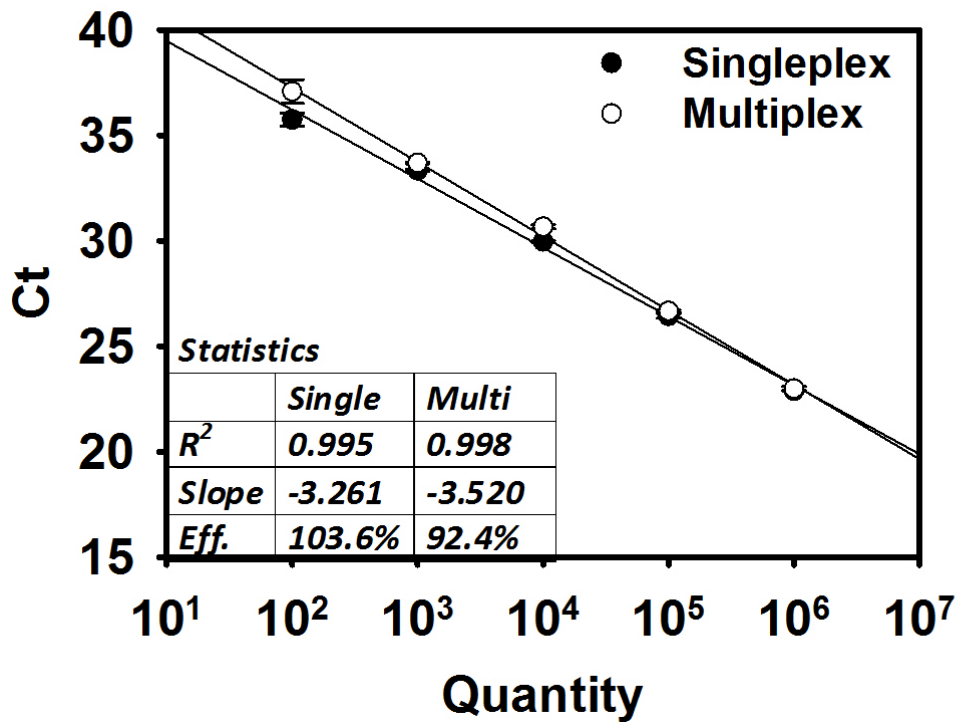
Quantitative PCR analysis for the lentiviral wild-type and control sequence (multiplex). Five 10-fold serial dilutions were used for the curve and the x-axis of the graph is the Ct values against the copy numbers on the y-axis. The Ct difference between them was less than one. The values of  $R^2$ , efficiency and slope fall within the standard required number.



**Fig 14: Standard calibration curve of lentiviral vector Wild-type Sequence.**

To validate the multiplex assay format, singleplex Vs Multiplex Ct values were compared for wild-type sequence which was analyzed on Q-PCR. The values of  $R^2$ , Efficiency and slope were almost the same for the wild type template in singleplex and multiplex with an average Ct difference of 0.14.



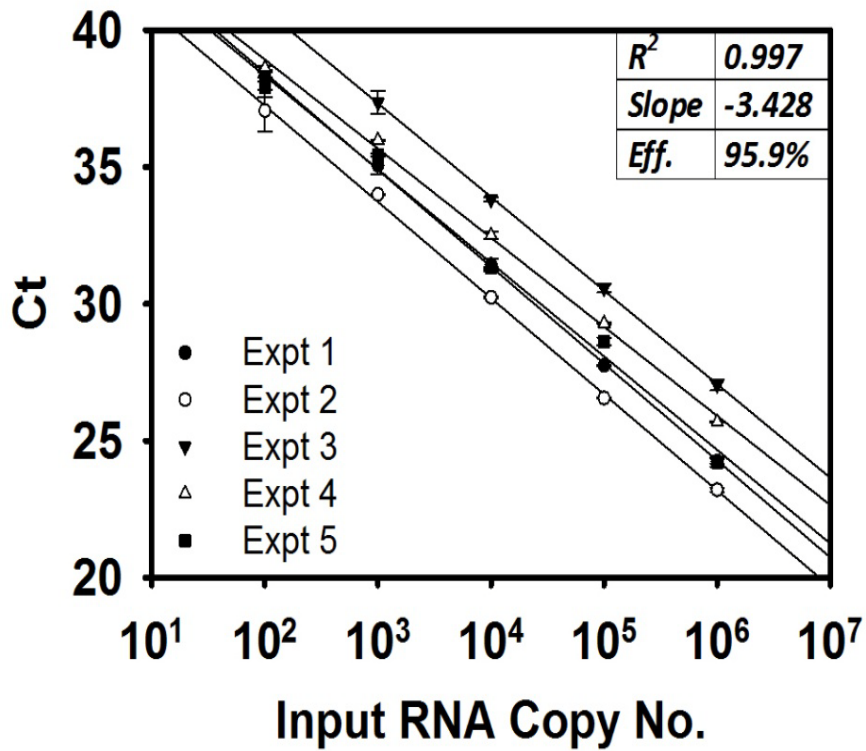


**Fig 15: Standard curve of lentivirus QC Sequence.**

To validate the multiplex assay format, singleplex Vs Multiplex Ct values were compared for control sequence which was analyzed on Q-PCR. The values of  $R^2$ , Efficiency and slope were close numbers in singleplex and multiplex and their average Ct difference between the two was 0.54.

### **Efficiency of Reverse Transcription-PCR:**

The 10-fold serial dilutions of RNA of quality control sequence were converted to cDNA by using High capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA). These cDNAs were amplified and quantified on Q-PCR with the primers/probe for the lentiviral vector QC. They were compared to the QC DNA standards (Fig 16). Five experiments were performed on five different days, and the efficiency of the reverse transcription kit ranged from 5% to 50% with an average of 20% (Fig 17).



**Fig 16: Standard curve of Lentiviral vector control sequence template-RNA.**

Comparison of Ct values for control sequence template-RNA that was run Singleplex. The y-axis being the number of RNA added before Reverse Transcription-PCR and the X-axis being the Ct values of cDNA amplified on Q-PCR after reverse transcription. The values of  $R^2$ , efficiency and slope are an average of five different experiments.

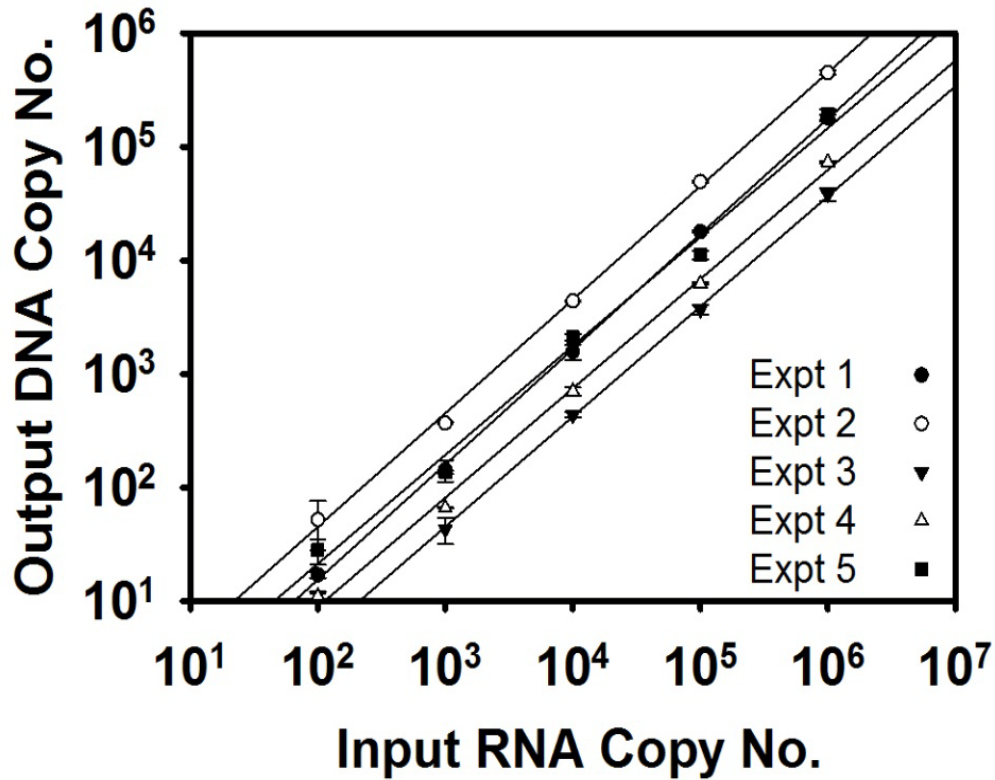


Fig 17: Comparison of the DNA copy numbers obtained from RNA after reverse transcription-PCR.

The output DNA copy number varied with each experiment. For an input of a 10000 copies the output varied from 5 to 5000 copies. The efficiency of reverse transcription-Q-PCR ranged from 5%-50% with an average of ~20%.

**Singleplex test on the recombinant adenovirus and lentivirus:**

The wild-type primers/probe were tested on the virus stocks by lysing them. Ten-fold serial dilutions of the adenoviral stock were lysed using proteinase K to disrupt capsid protein (Ma et al., 2001). They were compared to the wild type template standards on Q-PCR giving a total virus genome count that was 5 to 12-fold higher than the infectious titer.

The lentiviral vector was lysed using the detergent 0.5% Igepal-20 following a 10-fold serial dilution. They were compared to the standards by Q-PCR, giving a total virus genome count of  $3 \times 10^{11}$  per ml, which was 30,000-fold higher than the infectious titer.

**p24 assay:**

The p24 protein is the HIV viral capsid protein, and it was assessed using enzyme linked immunosorbent assay. The quantity of p24 revealed a total virus genome count of  $2.7 \times 10^{11}$  per ml, which was equivalent to the number obtained through by Q-PCR.

#### IV. DISCUSSION

The use of viral vectors for gene therapy has been studied widely. Viral vectors used in clinical trials, or in clinically infected animals, tend to distribute to non-target organs, followed by shedding (Delenda & Gaillard, 2005; Hackett et al., 2000; Heid, et al. 1996; Lizée et al., 2003). Although the vectors are generally safe, the potential risk of recombinant virus entering the environment needs to be assessed for proper biocontainment. Studies on the shedding of vector viruses are lacking in the scientific literature. In this study, a Q-PCR assay was developed to measure the amount of adenoviral and lentiviral vectors shedding from experimentally infected animals.

Adenoviral and retroviral vectors are the most commonly used vectors in gene therapy. The vectors in the current study were developed from commercially available kits. For *Adenovirus*, the infectious titer was determined by plaque assay and total virus genome count was determined by Q-PCR. From this, the ratio of total virus genome count (vg) to infectious viral particle count was calculated. This ratio reveals the quantity of dead or defective viral particles, which must be considered for any virus stock.

Noninfectious particles will contribute to the number of particles being shed from an animal.

In the current study, the adenoviral vector gave vg:pfu values of 12:1 and 5:1 for each of two stocks. Diaconu *et al* (2010) reported a similar ratio of 10:1 and 4.7:1 for a similar Ad5-based vector. In general, a high quality batch of the adenoviral vector would have a ratio less than 30:1 (Ludewig & Hoffmann, 2005).

For the lentiviral vector, the infectious titer was determined by colony forming unit (cfu) assay and total virus genome count was determined by Q-PCR. The ratio of vg:cfu for the lentiviral vector was 30,000:1, suggesting the presence of a large amount of defective particles. In general HIV genomes possess large proportion of defective viral particles and defective particles are mutants of the virus which might lack one or more gene (Bernier & Tremblay, 1995; Li *et al.*, 1991). The total virus genome count was confirmed by measuring concentrations of p24 Gag protein using enzyme-linked immunosorbent assay. The p24 assay revealed a vg:cfu ratio of 27,000:1. The correlation between viral titer and p24 protein has been studied. The p24 protein per particle number is converted to molecules

per virion by using a molecular weight of  $2.4 \times 10^4 \text{ g mol}^{-1}$  (Layne *et al.*, 1992).

The use of Real-time PCR for quantification of nucleic acids is an essential, powerful technique used in a wide range of analyses. Real-time PCR measures the amount of DNA produced in the exponential phase of amplification. In contrast, conventional PCR shows only the end-product of amplification, frequently in the plateau phase of amplification. This is why conventional PCR is not quantitative.

For the quantitative PCR assay in the current study, primers and probes were designed to amplify and detect the encapsidation region of *Adenovirus* type 5 and the RRE region of HIV-1, strain NL4-3. Because all generations of adenoviral and lentiviral vectors to date retain these regions, the primers and probes designed here can be universally used, assuming the serotype or strain is similar.

In order to assess the accuracy of the assay, the unknown sample is spiked with an internal control which helps the precise quantification. An external control that was amplified in a separate reaction would not be as accurate. A known number of molecules are added before the



processing of an unknown sample and during quantification, and it is co-amplified in a Q-PCR multiplex reaction. By using this strategy, the loss of sample during the processing is accounted for. The quality control sequence had minimal differences as compared to the wild type sequence to ensure similar efficiency for a comparable amplification. The primer binding regions remain the same for both wild type and quality control sequence. Saulnier *et al* (2003) reported the use of an internal control for adenoviral vectors in their study, but their control was  $\lambda$  bacteriophage for which primers and a probe were designed.

The quality control sequence to be spiked with the lentivirus was transcribed into RNA, which was then reverse transcribed into cDNA. The amplification curve was then compared to the curve for the DNA standards. In most cases, the efficiency of reverse transcription (cDNA synthesis) is often less than 100% and has been reported to be highly variable (Wong & Medrano, 2005). In the current study, cDNA was quantified using Q-PCR, and the efficiency of cDNA synthesis was found to be 20% on average. Since the genetic material of HIV-1 vector is RNA, it must be converted to cDNA for Q-PCR. Thus, this step is required, and the

conversion rate must be accounted for in the quantitative assay.

With a minimal number of sequence differences in the probe region of the wild type sequence, a quality control sequence was designed to spike the unknown samples.

Kawahira *et al.* (2010), in their study on viral shedding after adenoviral vector treatment for esophageal cancer, used conventional PCR to report only the positive or negative viral DNA in samples. The quantification of the sample was not performed to show the amount of virus the patient was shedding.

In the current study, five 10-fold serial dilutions of the vector DNA of wild type and quality control sequence were performed in singleplex reactions and optimized along with their specific primers and probes. The multiplex format showed that the quality control sequence did not interfere with amplification of the wild type DNA. This multiplex format makes use of two assay templates with two different probes and a single set of primers. Saulnier *et al* (2003) made use of the similar principle but they used completely different assay sets. The multiplex reaction in the current study clearly showed high specificity and sensitivity, to which the viral shedding can be compared.

The standard curves for assessing the wild type and quality control sequences were generated using a dynamic range of 4 logs ( $10^6$  to  $10^2$ ). The upper limit of this range was chosen based on the observation that Ct values were approaching the baseline Ct range (generally between cycles 3 and 15). The lower limit of the dynamic range was chosen based on the observation that Ct values for the  $10^2$  standard were less than or equal to 37. Values of Ct between 37 and 40 were defined as the equivocal range for this study. When limiting dilutions of DNA and RNA molecules for the adenoviral and lentiviral systems were performed and measured by Q-PCR, quantities below 10 (in the 37 to 40 Ct range) could be detected by extrapolation from the standard curve. The true limit of detection for viral shedding will be determined in future experiments using biological samples spiked with known amounts of virus.

#### **Conclusion and Future studies:**

In conclusion, a highly specific, sensitive, and reproducible assay using Q-PCR to quantify the shedding of adenoviral and lentiviral vector viruses from excretions of experimentally infected animals was developed. Future studies will include testing for replication competent virus (RCV) in the virus stocks. A multiplex reaction

performed on biological samples spiked with wild type virus and quality control sequences will confirm the sensitivity of the assay. Finally, testing samples obtained from infected and control research animals will reveal how much virus is being shed by specific routes of infection using specific doses for virus for the initial infection.

## V. GLOSSARY

BJ5183-AD-1: Electroporation competent bacterial cells

cDNA: Complementary deoxyribonucleic acid

CFU: Colony forming units

Ct: Threshold cycle

E1,E2,E3,E4: Early transcribed regions of adenovirus

*HIV-1: Human immunodeficiency virus-1*

PCR: Polymerase chain reaction

PFU: Plaque forming units

pShuttle-CMV-*lacZ*: Control shuttle vector

QC: Quality control

Q-PCR: Quantitative polymerase chain reaction

R<sup>2</sup>: Coefficient of determination

RRE: Rev response element

Wt: Wildtype virus

XL-10: Ultracompetent bacterial cells

## VI. REFERENCES

- Alemaný, R., Suzuki, K., & Curiel, D. T. (2000). Blood clearance rates of adenovirus type 5 in mice. *Journal of General Virology*, 81(11), 2605.
- Bernier, R., & Tremblay, M. (1995). Homologous interference resulting from the presence of defective particles of human immunodeficiency virus type 1. *Journal of virology*, 69(1), 291.
- Bett, A. J., Haddara, W., Prevec, L., & Graham, F. L. (1994). An efficient and flexible system for construction of adenovirus vectors with insertions or deletions in early regions 1 and 3. *Proceedings of the National Academy of Sciences*, 91(19), 8802.
- Delenda, C., & Gaillard, C. (2005). Real-time quantitative PCR for the design of lentiviral vector analytical assays. *Gene therapy*, 12, S36-S50.
- Dull, T., Zufferey, R., Kelly, M., Mandel, R., Nguyen, M., Trono, D., & Naldini, L. (1998). A third-generation lentivirus vector with a conditional packaging system. *Journal of virology*, 72(11), 8463.
- Gonin, P., & Gaillard, C. (2004). Gene transfer vector biodistribution: pivotal safety studies in clinical gene therapy development. *Gene therapy*, 11, S98-S108.

- GrisCELLI, F., Opolon, P., Saulnier, P., Mami-Chouaib, F., Gautier, E., Echchakir, H., Squiban, P. (2003). Recombinant adenovirus shedding after intratumoral gene transfer in lung cancer patients. *Gene therapy*, 10(5), 386-395.
- Hackett, N. R., El Sawy, T., Lee, L. Y., Silva, I., O'Leary, J., Rosengart, T. K., & Crystal, R. G. (2000). Use of quantitative TaqMan real-time PCR to track the time-dependent distribution of gene transfer vectors in vivo. *Molecular Therapy*, 2(6), 649-656.
- He, T. C., Zhou, S., Da Costa, L. T., Yu, J., Kinzler, K. W., & Vogelstein, B. (1998). A simplified system for generating recombinant adenoviruses. *Proceedings of the National Academy of Sciences*, 95(5), 2509.
- Heid, C. A., Stevens, J., Livak, K. J., & Williams, P. M. (1996). Real time quantitative PCR. *Genome research*, 6(10), 986.
- Hiltunen, M. O., Turunen, M. P., Turunen, A., Rissanen, T. T., Laitinen, M., Kosma, V., & Yla-Herttuala, S. (2000). Biodistribution of adenoviral vector to nontarget tissues after local in vivo gene transfer to arterial wall using intravascular and periadventitial gene delivery methods. *The FASEB Journal*, 14(14), 2230.

- Kawahira, H., Matsushita, K., Shiratori, T., Shimizu, T., Nabeya, Y., Hayashi, H., Shimada, H., (2010). Viral shedding after p53 adenoviral gene therapy in 10 cases of esophageal cancer. *Cancer science*, 101(1), 289-291.
- Kovesdi, I., Brough, D. E., Bruder, J. T., & Wickham, T. J. (1997). Adenoviral vectors for gene transfer. *Current opinion in biotechnology*, 8(5), 583-589.
- Layne, S., Merges, M., Dembo, M., Spouge, J., Conley, S., Moore, J., ...Nara, P. (1992). Factors underlying spontaneous inactivation and susceptibility to neutralization of human immunodeficiency virus. *Virology*, 189(2), 695-714.
- Li, Y., Kappes, J. C., Conway, J. A., Price, R. W., Shaw, G., & Hahn, B. (1991). Molecular characterization of human immunodeficiency virus type 1 cloned directly from uncultured human brain tissue: identification of replication-competent and-defective viral genomes. *Journal of virology*, 65(8), 3973.
- Lizée, G., Aerts, J. L., Gonzales, M. I., Chinnasamy, N., Morgan, R. A., & Topalian, S. L. (2003). Real-time quantitative reverse transcriptase-polymerase chain reaction as a method for determining lentiviral vector titers and measuring transgene expression. *Human gene therapy*, 14(6), 497-507.



- Ludewig, B., & Hoffmann, M. W. (2005). *Adoptive immunotherapy: methods and protocols* (Vol. 109): Humana Pr Inc.
- Ma, L., Bluysen, H. A. R., De Raeymaeker, M., Laurysens, V., van der Beek, N., Pavliska, H., . . . van Es, H. H. G. (2001). Rapid determination of adenoviral vector titers by quantitative real-time PCR. *Journal of virological methods*, 93(1-2), 181-188.
- Mittal, S. K., McDermott, M. R., Johnson, D. C., Prevec, L., & Graham, F. L. (1993). Monitoring foreign gene expression by a human adenovirus-based vector using the firefly luciferase gene as a reporter. *Virus research*, 28(1), 67-90.
- Miyoshi, H., Blomer, U., Takahashi, M., Gage, F. H., & Verma, I. M. (1998). Development of a self-inactivating lentivirus vector. *Journal of virology*, 72(10), 8150.
- Pan, D., Gunther, R., Duan, W., Wendell, S., Kaemmerer, W., Kafri, T., Whitley, C. B. (2002). Biodistribution and toxicity studies of VSVG-pseudotyped lentiviral vector after intravenous administration in mice with the observation of in vivo transduction of bone marrow. *Molecular Therapy*, 6(1), 19-29.

- Russell, W. (2009). Adenoviruses: update on structure and function. *Journal of General Virology*, 90(1), 1.
- Stratford-Perricaudet, L., Makeh, I., Perricaudet, M., & Briand, P. (1992). Widespread long-term gene transfer to mouse skeletal muscles and heart. *Journal of Clinical Investigation*, 90(2), 626.
- Tiesjema, B., PH Hermsen, H., CH van Eijkeren, J., & FA Brandon, E. (2010). Effect of administration route on the biodistribution and shedding of replication-deficient HAdV-5: a qualitative modelling approach. *Current gene therapy*, 10(2), 107-127.
- Verma, I. M., & Weitzman, M. D. (2005). Gene therapy: twenty-first century medicine. *Annu. Rev. Biochem.*, 74, 711-738.
- Volpers, C., & Kochanek, S. (2004). Adenoviral vectors for gene transfer and therapy. *The Journal of Gene Medicine*, 6(S1), S164-S171.
- Warnock, J. N., Daigre, C., & Al-Rubeai, M. (2011). Introduction to Viral Vectors. *Methods in molecular biology (Clifton, NJ)*, 737, 1.
- Wong, M. L., & Medrano, J. F. (2005). Real-time PCR for mRNA quantitation. *Biotechniques*, 39(1), 75.
- Wood, M., Perrotte, P., Onishi, E., Harper, M. E., Dinney, C., Pagliaro, L., & Wilson, D. R. (1999).

Biodistribution of an adenoviral vector carrying the luciferase reporter gene following intravesical or intravenous administration to a mouse. *Cancer gene therapy*, 6(4), 367.

Zhang, Y., & Bergelson, J. M. (2005). Adenovirus receptors. *Journal of virology*, 79(19), 12125.

Zhao, Z. Z., Liu, W., Su, Y. X., Zhu, J., Zheng, G. H., Luo, Q., & Jin, X. Q. (2010). Research Evaluation of biodistribution and safety of adenovirus vector containing MDR1 in mice. *drugs*, 7, 8.

Zufferey, R., Dull, T., Mandel, R. J., Bukovsky, A., Quiroz, D., Naldini, L., & Trono, D. (1998). Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. *Journal of virology*, 72(12), 9873.