DEVELOPMENT OF A RAPID FLUORESCENCE-BASED ADENOVIRUS INACTIVATION ASSAY

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ABSTRACT

Screening of potential antiviral product prototypes is typically performed using The American Society for Testing and Materials (ASTM) method E1052 “Efficacy of Antimicrobial Agents against Viruses in Suspension.” Adenovirus (ADV) is a recommended test virus and demonstrates intermediate resistance to chemical inactivation. The procedures commonly employed to quantify infectious virus are median cell culture infective dose (CCID50) and the plaque assay (PA). The rate-limiting step in both of these procedures is the time needed to form observable cytopathic effect (CPE) in infected cells, which takes approximately 7 days for ADV. Virus inactivation studies were performed according to ASTM E1052 using the traditional PA method as well as an experimental fluorescence-based method utilizing a recombinant ADV (ADV-GFP) that constitutively expresses green fluorescent protein (GFP). Infective units were determined by counting the number of fluorescent green cells or plaques. The $\log_{10}$ reduction (LR) values of virus inactivation were determined for several different concentrations of ethanol and hypochlorite using the traditional PA method and the ADV-GFP method. The LR values using the ADV-GFP method were similar, within +/- 0.5 $\log_{10,}$ to the LR values obtained using the traditional PA method. The ADV-GFP method was shown to be an acceptable substitute yielding results in 2 days vs. 7 days for the traditional methods. In addition, A549 cells were stably transfected with a plasmid construct containing a GFP gene under control of the ADV early gene E2 promoter for the eventual use in a newly conceived assay for quantitating ADV infection.

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List of Symbols and Abbreviations

ADV	Adenovirus
AOAC	Association of Official Analytical Chemists
ASTM	The American Society for Testing and Materials
CCID50	Median Cell Culture Infective Dose
CDC	Centers for Disease Control and Prevention
CPE	Cytopathic Effect
DMEM	Dulbecco's Minimal Essential Media
DPBS	Dulbecco's Phosphate Buffered Saline
EBSS	Earle's Balanced Salt Solution
EPA	Environmental Protection Agency
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
GFP	Green Fluorescent Protein
HBSS	Hanks Balanced Salt Solution
HCM	High Count Method
hrGFP	Humanized Renilla Green Fluorescent Protein
LCM	Low Count Method
LR(s)	Log_{10} Reduction(s)
MEM	Minimal Essential Media
MOI	Multiplicity of Infection
PA	Plaque Assay
PFU	Plaque Forming Units

RT	Room Temperature
TK	Time-kill
wt	Wild Type

Chapter 1

INTRODUCTION

Section 1.1 Antiseptics and Disinfectants

Inanimate environmental surfaces and human hands are common vectors enabling the transmission of pathogenic microorganisms. One effective infection prevention and control tactic is to employ antimicrobial products to destroy the organisms before they can be transferred. Antiseptics are antimicrobial washes and sanitizers that are used on the skin, while disinfectants are products used on inanimate surfaces [1]. Over 5000 different antimicrobial products are currently marketed in the United States and over one billion dollars are spent each year on these products, more than half of which is in the health care industry [2]. In the United States most disinfectant products are classified as pesticides and are regulated by the Environmental Protection Agency (EPA), however, many antiseptic products are classified as drugs and are regulated by the Food and Drug Administration (FDA) [3]. Both regulatory agencies require products to meet defined minimum antimicrobial efficacy based on the intended use of the product. There are many different classes of antiseptic and disinfectant products ranging from mild sanitizers designed to slightly decrease the numbers of some organisms to solutions that are used to sterilize reusable medical equipment. The specific method, organisms and pass/fail criteria required to demonstrate antimicrobial efficacy vary for each product class and determine the antimicrobial statements that the manufacturer can claim on the product label.

Enveloped viruses and vegetative bacteria are relatively easy to kill while bacterial endospores are at the other end of the spectrum and are extremely difficult to kill. In fact, the definition of sterilization is the complete kill of bacterial endospores. Non-enveloped viruses, fungi, and mycobacteria fall somewhere in between. The FDA, EPA, and Centers for Disease Control and Prevention (CDC) specify minimum requirements for disinfectant products based on the risk of infection transmission for a given application. High-level disinfection is required for critical items such as endoscopes, while only minimal efficacy is required for general use such as wiping the outside of a stethoscope. Figure 1.1 illustrates this hierarchy of chemical germicides [4].

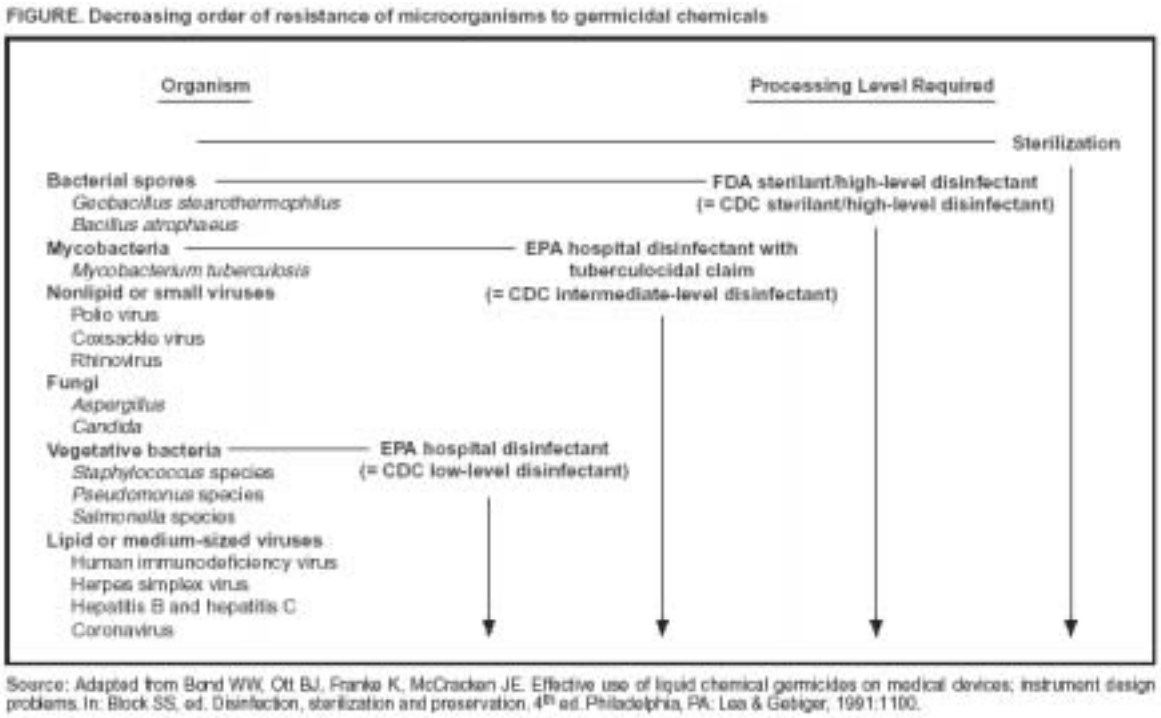


Figure 1.1 Classes of Surface Disinfectants

Section 1.2 Virucidal Product Development

Infections and illnesses caused by non-enveloped viruses are a significant public health concern. These viruses can persist in the environment and be transmitted from person to person via surfaces, fomites, and hands [5]. Hand transmission has been shown to be a contributing factor in rhinovirus upper respiratory infections [6] as well as in adenovirus (ADV) infections. There are numerous strains of ADV that cause a variety of illnesses including conjunctivitis, pneumonia, and gastroenteritis [7]. Norovirus, which is estimated to cause more cases of foodborne disease than any other organism in the United States, [8] can be transmitted by both hard surfaces and hands [9]. In addition, many serious illnesses and infections are the result of hepatitis A viruses and rotaviruses which have both been shown to be transmitted by surfaces and hands [10,11]. Products that are highly effective at inactivating these clinically relevant viruses on animate and inanimate surfaces are a valuable infection control tool.

There are many antiviral surface disinfectant products on the market in the US which are registered through the EPA. In contrast, the FDA does not yet have a routine process for evaluating antiviral efficacy or making antiviral claims for antiseptic products. There are currently no products on the market approved by the FDA for use in an antiviral antiseptic application. To do so would require approval through the new drug application process. Unfortunately, this route can be relatively costly and slow rendering it too risky and impractical for many companies. This situation has the negative effect of discouraging innovation of new antiviral antiseptic formulations [12]. Many leaders in the field believe that it would be a significant benefit to public health if more antiseptic development companies improved the antiviral efficacy of available products. They feel

that it is important for the US FDA to make it easier for companies to develop antiviral antiseptic products. If the FDA decides to move in this direction, one of the first hurdles it will face will be to define the required standard test methodologies. It is, therefore, potentially a significant benefit for researchers to develop more efficient and effective antiviral assays [7,13].

Development of a virucidal disinfectant or antiseptic product involves several steps. Possible new antiviral formulations are initially screened using an *in vitro* suspension test, often referred to as a time-kill (TK) test. TK procedures measure how well a test formulation inactivates or kills a specific challenge microorganism in a certain period of time. In antiviral TK testing methods, a suspension of the target virus in the test formulation is created and allowed to react for the duration of the exposure time. The surviving number of infectious virus particles, e.g. plaque forming units (PFU), is compared to the initial virus titer [3]. Results are reported in terms of $\log_{10}$ reduction (LR), where a 1 LR means that 90% of the organisms were killed, and a 2 LR means that 99% were killed, etc. If a formulation performs well in the TK test it will then undergo more rigorous surface-based testing relevant to the intended application of the product being developed. These tests will determine if the test formulation is able to kill and/or remove the virus on a surface such as skin or stainless steel. Log_{10} reductions (LRs) observed for a given test formulation using surface-based tests are typically much lower than those obtained using the screening suspension tests. Efficacy against viruses on surfaces is more challenging since the product must penetrate the dried matrix containing the virus and all surfaces of the organism are not necessarily in contact with the solution. The surface may also contain other interfering organic or inorganic substances that can

inactivate the antimicrobial activity of the formulation. For these reasons, TK suspension tests used in early screening are not highly predictive of how well the product will actually perform for its intended application [7,14]. Several standard method guidelines used in the development of virucidal products are published by The American Society for Testing and Materials (ASTM) and the Association of Official Analytical Chemists (AOAC). Figure 1.2 illustrates the flow of virucidal product development while highlighting a few sample standard virucidal test methods [15-18].

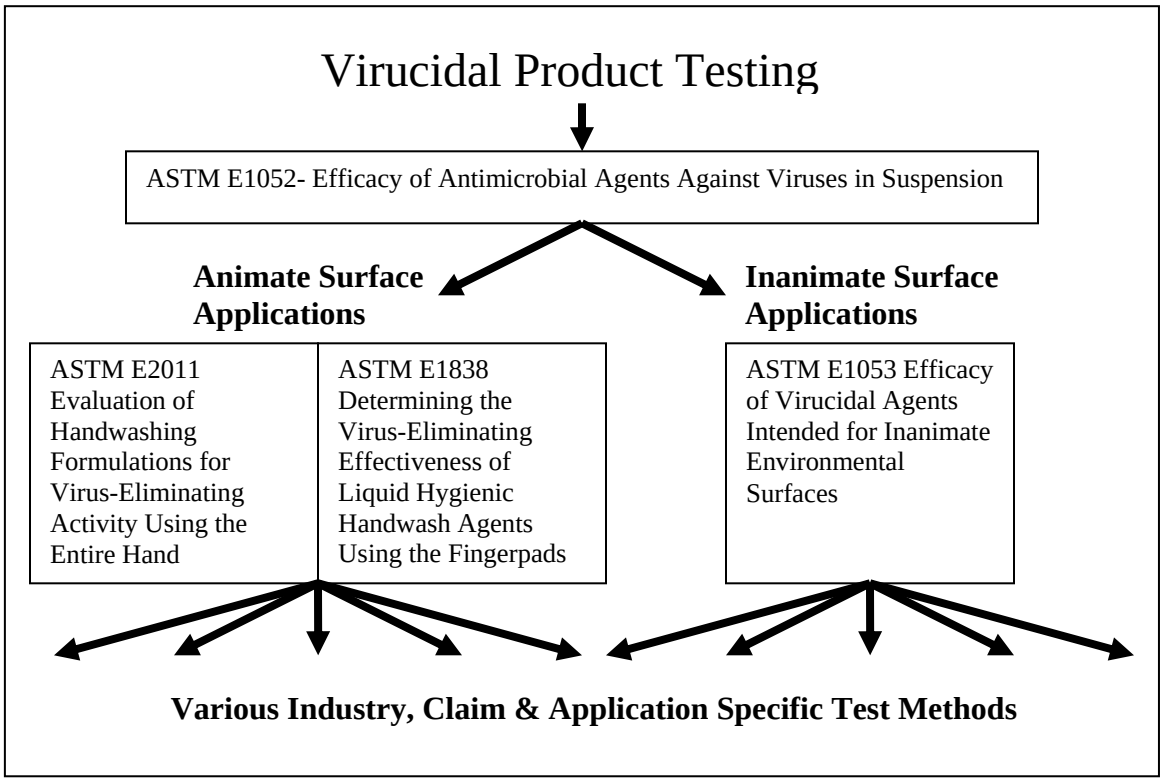


Figure 1.2 Virucidal Product Development Testing Methods

Section 1.3 Antiviral Suspension Assays

ASTM E1052 "Efficacy of Antimicrobial Agents Against Viruses in Suspension" is a published standard method that is commonly used as a guideline for those who

conduct antiviral suspension TK testing. The ASTM virus TK method involves exposing the virus to the test product solution followed by quenching of the reaction at a specified time point by dilution and/or use of a chemical neutralizer [15]. The neutralization step is critical to avoid false positive results. If the activity of the antimicrobial agent is not stopped at the specified exposure time point, the observed LR may appear to be artificially high. The effectiveness of the neutralization method employed must be verified for every formulation tested in order to obtain accurate results [3]. ASTM methods E1054 and E1482 explain how to appropriately conduct a neutralization effectiveness test. Briefly, the test sample and the neutralization solution are combined in the ratio that will occur during the TK test then the virus is added to that mixture and quantified. The neutralization is considered effective if the virus titer is not significantly reduced by exposure to the neutralized test formulation. Some formulations such as high-level disinfectants can be extremely difficult to neutralize. In these cases, a gel exclusion column can be employed to rapidly separate the virus from the test solution [19,20]. The active ingredients and concentrations employed in antiseptic formulations, however, like those that were tested in this project, can usually be effectively neutralized by dilution into a neutralizing solution.

After neutralization of the reaction, the sample is diluted and analyzed using a quantifiable measure of infectivity. Viral identification methods based on quantification of the number of viral particles, antigen-binding, or detection of viral genetic material are not acceptable for use in TK assays because the mechanisms of kill of various antimicrobials may disrupt the particle but not the component that is detected or leave the particle intact yet unable to infect a cell. Therefore, virus detection methods such as

spectrophotometric determination, antibody-binding or detection of genetic material are often inappropriate.

In addition to the neutralization control test, there are three other controls that are necessary to validate results when performing an antiviral TK test. First, there must be a cell culture control which has at least one well in each cell culture plate that is not manipulated. Observation of healthy cells in these wells provides assurance that the cells were acceptable for use and that the reagents and cells were not contaminated. The second control is the virus control which serves as the virus titer. In this set of wells the virus is not exposed to the test sample and therefore the number of infectious particles is equal to the amount that one started with. Cytotoxicity of the test sample on the cells is measured by the third control. If the test sample itself kills the cells, the remaining virus will not be able to infect the cells and one will not be able to determine the amount of remaining virus. In the cytotoxicity control, virus is not added. The test sample is inoculated with an equal volume of solution without virus but otherwise treated the same as the test runs [14].

In practice, most laboratories performing ASTM E1052 and similar virucidal TK methods use one of two ways to quantify infective virus, median cell culture infective dose (CCID50) or the plaque assay (PA). Both methods involve observation of the cytopathic effect (CPE) on cells due to virus infection. The major difference is that in the PA the cells are covered with a dense overlay that doesn't allow virus to spread through the media which results in clear zones of cell death, plaques, while in CCID50 the virus produced from an infected cell can travel freely in the media and infect cells elsewhere in the well. For CCID50 but not for counting plaques, an average of the overall amount of

CPE is noted for all the cells in the tissue culture well. High throughput commercial testing labs rely almost exclusively on the CCID50 method because it requires less total hours of labor, plates and reagents than the PA. The PA method, however, is more precise, more quantitative, and more accurate than the CCID50 method, but since it is more difficult to perform it is generally used only in research laboratories [14]. The commercial laboratories compensate for the decreased precision and accuracy inherent in the CCID50 methodology by obtaining results averaged from replicate trials.

Section 1.4 Adenovirus Time-Kill Methodology

ASTM E1052 suggests testing formulations using 10 different viruses that represent the range of antimicrobial susceptibility from the very easy to kill enveloped viruses, like herpes simplex and influenza, to the notoriously difficult to kill, poliovirus [15]. In practice, though, cost-effective product development necessitates judicious selection of a minimal subset of organisms [21]. ADV Type 2, Adenoid 6 strain is one of the suggested viruses with intermediate susceptibility [15]. If a product performs well against ADV it would also perform very well against the easier to kill viruses. On the other hand, if a product has no activity against ADV, it would likely not have any activity against the more difficult to kill viruses. ADV therefore is particularly well suited for use in the earliest stages of product development when the efficacy level of test formulations is unknown [21]. Unfortunately, it takes 2-3 times longer to get results from an ADV TK test than it does for most of the other viruses suggested by this method. This makes ADV a less desirable choice for initial screening since turn around time for results is usually of utmost importance during the early screening stages of new product development.

There are many limitations and disadvantages of the traditional ADV TK methods, CCID50 and PA. The most significant disadvantages of these methods are their reliance on observation of CPE as evidence of infection. Results from an ADV TK assay using traditional CPE-based methods cannot be obtained in less than 1 week, because it takes ADV at least that long to kill enough infected cells to result in visible CPE. In addition, both PA and CCID50 methods require the technician performing the assay to use their judgment during manual collection of data. The accuracy of results obtained can easily be adversely affected by inadequate technician training or inadvertent human error. The CCID50 method, in particular, requires a great deal of expertise and familiarity with tissue culture cells to accurately and consistently perform the subjective grading of the appearance of CPE in cells.

Both traditional method variations have several other disadvantages in addition to slow turn around time and a dependence on the skill of the technician. For one, neither the CCID50 nor the PA method can be easily automated. The major obstacle to automation of the CCID50 procedure is the ability of an instrument to observe and grade cells for the appearance of CPE. One would need to employ additional steps to include an indicator of cell death or growth that could be measured by instrumentation. In the PA, it is a challenge to count plaques of variable size and differing staining contrast from run to run. Most importantly, the precision of CCID50 results is poor. Even after performing this method in quadruplicate, the results can only be resolved to increments of 0.25 log. The PA method yields more precise results than the CCID50 method since it is a more direct measure of the number of infectious virus particles, but it has the major

disadvantage of being significantly more labor intensive and of requiring many more manipulations and materials to perform.

Chapter 2

PROBLEM STATEMENT & PROPOSED SOLUTIONS

Screening of new formulations for anti-ADV efficacy using CCID50 or PA methods is neither trivial nor is it well-suited for conversion to a high-throughput automated process. The purpose of this research project was to develop an alternate methodology that would yield results equivalent to the traditional methods but in less time and in a manner that is more conducive to automation.

In order to significantly improve the ADV TK assay, it was necessary to circumvent the traditional assays' reliance on observation of CPE. We chose to pursue a more rapid indicator of infectivity that could be objectively quantified with existing instrumentation. As a result, two new method variations were conceived that would measure fluorescence as an indicator of infection based on the utilization of virus induced expression of green fluorescent protein (GFP). The first method involved replacing *wt* (wild type) ADV with recombinant ADV that constitutively expresses GFP (ADV-GFP) upon infection of a complementary cell line, HEK293. The second method involved engineering cells to express GFP upon infection with *wt* ADV.

Both of the proposed GFP-based alternate methods have several advantages over the current CPE-based PA and CCID50 procedures. The most significant advantage is that they reduce the total time to complete an assay from 7 or more days to only 1-2 days. Since GFP expression occurs only when the virus replicates, the appearance of a green cell in either method indicates that the virus has successfully infected the cell and thus can be used as a measure of infectivity instead of waiting for the appearance of

subsequent viral-induced CPE. The accuracy of this counting method would be similar to that obtained in the PA but without the need for staining or other additional manipulations that are required for plaque visualization. Thus both proposed solutions have the potential to be as accurate as the PA method while being as easy to perform as the CCID50. In addition, measurement of fluorescence in place of CPE reduces the assay's dependence on a technician's skill by increasing objectivity during data collection as well as increasing the feasibility of automation.

The creation of a cell line that expresses GFP upon *wt* ADV infection is the preferred method of the two proposed solutions. For one, the ADV-GFP is not the ASTM recommended *wt* virus strain. Use of the recombinant ADV-GFP could create issues because results with this virus might not always directly compare to the standard strain for which many companies may have historical data. There is also the possibility that the recombinant virus is significantly different than the *wt* one and that the results might not be as clinically relevant. Unlike the ADV-GFP method, the new cell line could be used with many *wt* ADV strains including the standard strain. Table 2.1 summarizes the advantages and disadvantages of the two new proposed methods as well as the traditional methods.

Table 2 Comparison of Proposed Methods to Traditional Ones

	Traditional Methods		New Ideas	
	CCID50	**PA**	**ADV-GFP**	**New Cell Line**
Days for Results	7-10	7-10	1-2	1-2
Supplies Cost	low	high	low	low
Time to Perform	med	long	short	short
Relative Skill Required	high	med	low	low
Accuracy and Precision	poor	good	good	good
Follows ASTM E1052	yes	yes	no	yes
Multiple Strains	yes	yes	no	yes
Automatable	unlikely	unlikely	possible	possible

Chapter 3

MATERIALS & METHODS

Section 3.1 Cells and Virus

HEK293 cells (human embryonic kidney; ATCC CCL-1573) and A549 cells (human carcinoma; ATCC CCL-185) were maintained in minimal essential medium (MEM), (Invitrogen) containing 10% fetal bovine serum (FBS). HEK293 cells are E1-complementing and support the replication of E1-deleted replication-defective ADV. HeLa cells (human cervical carcinoma; ATCC CCL-2) were maintained in Dulbecco's MEM (DMEM) with 10% FBS. Vero cells were maintained in 199 media with 5% FBS. All media were supplemented with penicillin (50 U/mL), streptomycin (50μg/mL), and 2.25 mM $NaHCO_3$. All cells were grown at 37 °C with 5% CO_2 and passaged 1 – 2 times per week. Cells were preserved in a DMSO-containing cryoprotectant solution at -140 °C.

ADV serotype 2 (ATCC VR-846) is a *wt* virus originally isolated from an infected individual with hypertrophied tonsils and adenoids. ADV-GFP was obtained from Vector Biolabs and is a $E1^-$ $E3^-$ replication-defective, type 5 recombinant virus containing a GFP reporter gene in the E1 position under control of a cytomegalovirus promoter. All experiments conducted using ADV-GFP were performed in HEK293. Preparation of viral stock was performed in MEM with 1% FBS. Cells (HeLa in earlier experiments, A549 in later experiments) were grown to 70 – 90 % confluence and virus was added such that the multiplicity of infection (MOI) was approximately 10. The cells were incubated 4 – 7 days then frozen at -70 ℃ overnight. After 3 – 4 freeze-thaw cycles,

the resulting solution was aliquoted into cryovials and stored at -70 °C. Some preparations were centrifuged to remove the cell debris prior to freezing.

Section 3.2 Adenovirus Time-Kill Testing

A 100 µL aliquot of virus-containing inoculum was added to 900 µL of the test sample. After a specified exposure time, ranging between 15 s - 60 s depending on the specific experiment, a 100 µL aliquot of the challenged sample was dispensed into 900 µL of a neutralizing solution (which was 1% Sodium Thiosulfate with or without 50% FBS in earlier experiments and 100% FBS in later experiments) and serially diluted into a buffer solution (Hank's Balanced Salt Solution, HBSS, in earlier experiments and Earle's Buffered Saline Solution, EBSS, in later experiments). For the purposes of determining the initial titer of the virus inoculum, each test run had at least one trial in which the test sample was a negative control without antiviral activity (water, Dulbecco's Phosphate Buffered Saline (DPBS), HBSS, or EBSS). These same initial steps were utilized regardless of the method used to quantify the amount of infectious virus, PFU/mL. All PFU/mL values were converted to their $\log_{10}$ value and LRs were calculated by subtracting the log of remaining virus from the log of the initial viral titer.

Section 3.3 Adenovirus Quantification

Section 3.3.1: PA

A 100 µL aliquot from each of the neutralized TK reaction serial dilutions was plated in duplicate onto confluent A549 cells in 12-well tissue culture plates after removal of the growth medium. The plates were swirled periodically during 60 min of

incubation at 37 ºC then covered with an overlay of 2 mL of MEM containing molten 0.6% agarose. Plates were incubated for 7-8 days to allow clear zones of cell death, plaques, to form at which time 2-3 mL of a 4% formaldehyde solution in physiological saline was added above the agarose plugs and allowed to soak at room temperature overnight. Plaques were counted after the agarose plugs were removed under running cold water and the cells stained with a 1% crystal violet solution and dried.

Section 3.3.2 CCID50

Monolayers of A549 cells were grown in 24, 48 or 96 well tissue culture plates. Just prior to the test, the cells were given fresh media which contained reduced amounts of FBS (1 – 2% vs. 5 – 10% in the original growth media). The TK assay was performed and 50-200 µL of the resulting dilutions were added directly to the wells. The plates were incubated for 7-10 days and each well was observed for quantity of CPE. Wells showing >50% of the cells with CPE were labeled as "+" while wells with <50% CPE were labeled as "0". If cytotoxicity control wells showed evidence of cytotoxicity they were labeled as "T". LRs were calculated using the Spearman-Karber formula:

$$M = xk + d\,[0.5 - (1/n)(r)]$$

$M = \log_{10}$ virus

xk = the number of the last dilution plated relative to the initial virus inoculum

(a 6 if 10^{-6} was the most dilute tube used)

d = log of the spacing between dilutions (a 1 if doing 10-fold dilutions)

n = number of replicates tested

r = number of wells that were labeled as "0"

Table 3.3.2 shows an example of the application of this formula to a sample data set.

Table 3.3.2 CCID50 Calculation Example

Dilution	Virus Control	Product A	Product B
-2	+ + + +	T T T T	+ + + +
-3	+ + + +	0 0 0 0	+ + + +
-4	+ + + +	0 0 0 0	+ + + +
-5	+ + + +	0 0 0 0	+ + + +
-6	+ + + +	0 0 0 0	+ + 0 +
-7	+ + + +	0 0 0 0	0 0 0 0
-8	0 0 0 0	0 0 0 0	0 0 0 0
-9	0 0 0 0	0 0 0 0	0 0 0 0
$CCID_{50}$	$10^{7.5}$	$\leq 10^{2.5}$	$10^{6.25}$
LR	NA	≥5.0	1.25
Spearman-Karber formula: M = xk + d [0.5 - (1/n)(r)] e.g. Product B: M_B = 9 + 1 [0.5 - (1/4)(13) = 6.25 Log Reduction of Product B = M_{VC} -M_B = 7.5 - 6.25 = 1.25			

Section 3.3.3 ADV-GFP

A 100 µL aliquot from each of the TK serial dilutions was plated in duplicate onto nearly confluent HEK293 cells in a 24-well tissue culture plate and incubated for approximately 30 – 50 hours. Each well was examined for the presence of green cells using fluorescence microscopy at 10X or 20X magnification. The total number of green cells in each well was calculated by counting a portion of the well and multiplying the number of observed green cells by a factor to account for the total area of the well vs. the area counted. A larger area was counted in wells that had lower counts of green cells while a smaller area was counted in wells that contained relatively large amounts of green cells. These two counting methods and their associated calculations were called the high count method (HCM) and low count method (LCM) and the details of the associated calculation are explained in Appendix I.

Section 3.4 Engineering New Cell Line

Section 3.4.1 DNA Purification and Concentration Determination

All ADV DNA used for PCR or enzymatic reactions was purified using the Promega Wizard kit (Cat#A1125) according to the manufacturer's directions. Plasmid preparations were purified using Sigma's Genelute plasmid mini-prep kit (PLN 70). DNA concentration measurements were obtained with a ND-1000 nanodrop spectrophotometer (Nanodrop Technologies, Inc.).

Section 3.4.2 E2 Promoter Amplification, PCR and Electrophoresis

The ADV E2 early gene promoter was amplified by PCR using primers designed to flank the published sequence of the E2 gene promoter region [22]. PCR was performed using a BioRAD DNA Engine. The PCR amplification program used began with 5 min at 96 ºC followed by 30 cycles of 1 min 95 ºC, 1 min 60 ºC, 1 min 68 ºC and finishing with 10 min at 68 ºC. Restriction sites for Hind III and Bam HI were incorporated into the primers for the eventual insertion into a vector containing the same restriction sites. Figure 3.4.2 illustrates the E2 promoter target sequence and the primers used. Amplification of the E2 sequence was verified by gel electrophoresis using a 2% agarose gel.

Ad E2 promoter region (#27078-27200, 123 bp)

27078→ 5′- ac / gcgga ggctc / tct*tc agcaa / atact gcgcg / ctgac tctta /
(HindIII) 3′- **Tg / cgcct ccgag / aga*ag t**cgtt / tatga cgcgc / gactg agaat /
5′- *ggAAG CTTac / gcgga ggctc / tct*tc a* – 3′

aggac tagtt / Tcgcg ccctt/ tctca aattt / aagcg cgaaa / actac gtcat /
tcctg atcaa /Agcgc gggaa/ agagt ttaaa / ttcgc gcttt / tgatg cagta /

3′- **tg ggccg / cggtc gtgga / c**CCTA GGgg -5′
ctcca gcggc / Cac**ac ccggc / gccag cacct / g** -3′ ← 27200
gaggt cgccg / Gtgtg ggccg / cggtc gtgga / c -5′ (BamHI)

Figure 3.4.2 ADV E2 Promoter Sequence and Primers

Section 3.4.3 Plasmid Preparation, Transformation and Selection

A promoterless mammalian expression vector containing humanized Renilla GFP (hrGFP), phrGFP, *cre* recombinase and the pExchange eukaryotic resistance module EC-Neo were obtained from Stratagene. Figure 3.4.3 illustrates the vector and its multiple cloning site region that was used as the point of insertion for the E2 promoter fragment. Restriction enzymes Hind III and Bam HI were used to treat the phrGFP plasmid as well as the E2 promoter fragment. T4 DNA Ligase was used to combine the fragments. The resulting plasmid was transformed into competent DH5 *Eschericia coli* cells (Invitrogen) and plated onto Luria Broth agar plates containing 100 U/mL ampicillin. Several colonies were selected and colony PCR was performed using the E2 promoter primers to detect the presence of the E2 promoter sequence in the DNA. A plasmid preparation containing the E2 sequence from a colony exhibiting ampicillin resistance was reacted with the Stratagene phrGFP shuttle vector containing neomycin resistance using *cre* recombinase. The site specific *cre* recombinase catalyzes the exchange of DNA located between the

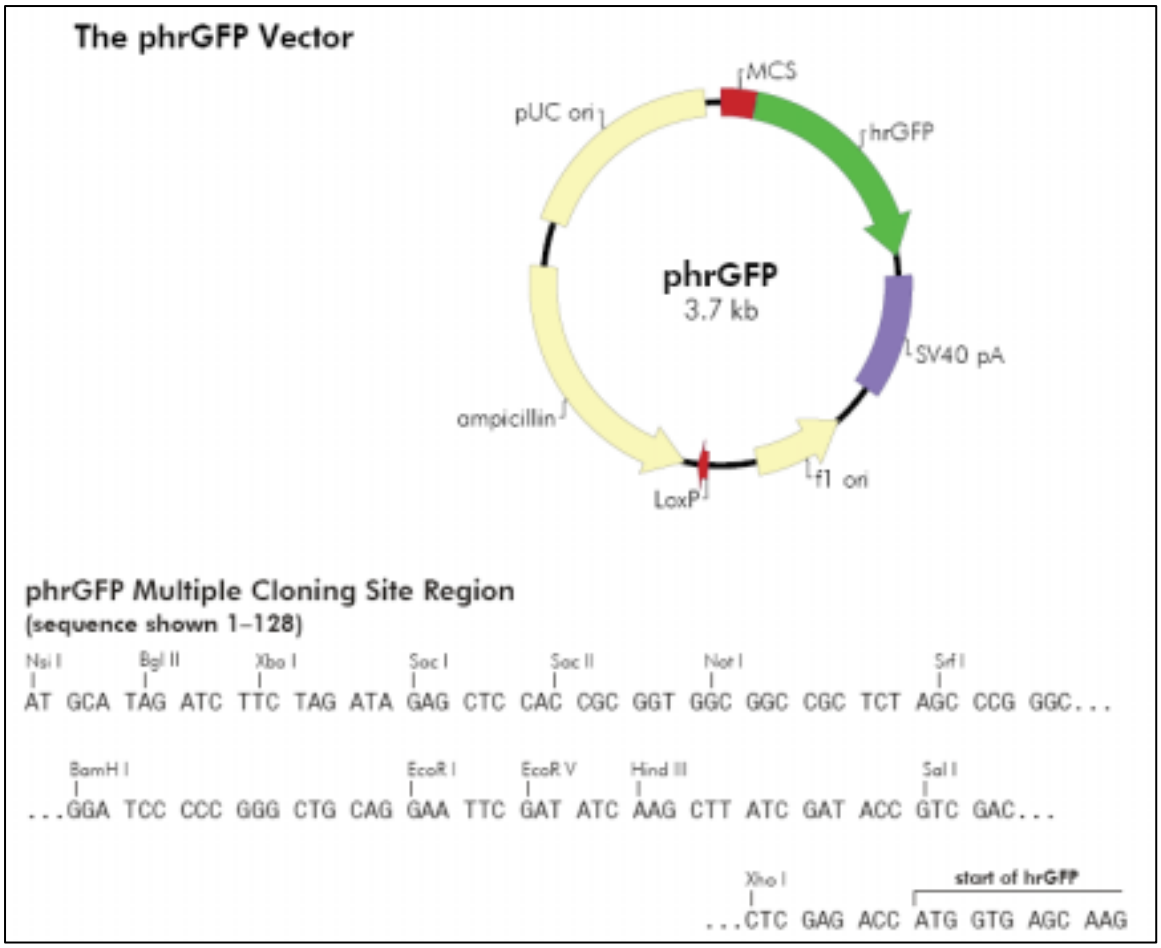


Figure 3.4.3 Features of the Stratagene phrGFP Vector

specific recognition sequences. The recognition site (LoxP) consists of two inverted 13-bp repeats and an intervening, 8-bp nonpalindromic region. Intermolecular and intramolecular recombination between the two sites can occur. Addition of the neomycin resistance gene was necessary to allow for selection in eukaryotic cells in upcoming stages of the cell line production. After insertion of the neomycin resistance gene, *E. coli* was transformed with the new plasmid and colonies containing the plasmid were selected with both ampicillin and kanamycin on Luria Broth agar plates. The DNA from some of the colonies was tested for the presence of the E2 promoter region using colony PCR.

This process was repeated twice yielding two batches of plasmids. A summary of all of the plasmids created throughout this process are described in Table 3.4.3. Plasmids were named according to the date they were produced and given alphanumeric codes based on their colony and plate of origin during the transformation selection steps. The final plasmid construct used to create the final cell line was designated as pCZ479. Some of the plasmid preparations with similar properties were pooled together and used for future transfection experiments. In hindsight, it was advised that pooling of different plasmid preparations is not desired, but at the time this was done with the intention of ensuring that if some of them were functional and others were not, the pooled mix would at least contain some functional ones.

Section 3.4.4 Transfection

Transfection of eukaryotic cells was carried out using lipofectamine reagent in combination with PLUS Reagent (Invitrogen). Several different transfection conditions were tested in order to determine the optimal conditions to achieve the highest possible transfection efficiency. The standard procedure that was used involved using 2 µL of lipofectamine, 400 ng of DNA, 2 µL of PLUS reagent and allowing the transfection reaction to proceed for 4 hours. During optimization, varying ranges of lipofectamine (1-4 µL), DNA (200 – 800 ng) and and transfection times up to 15 hours were tested. Transfection efficiencies were calculated by dividing the number of GFP expressing fluorescent cells by 10,000 (which was the approximate number of cells in each well) and then multiplying by 100%.

Table 3.4.4 Plasmid Descriptions

Plasmid Name	E2 Promoter	NEO Resistance	Description
phrGFP	**N**	**N**	Original unmodified promoterless plasmid from Stratagene
E10-012107	Y	**N**	
D8-012107	Y	**N**	
D9-012107	Y	**N**	
E6-012107	Y	**N**	
E8-012107	Y	**N**	
E2promMIX	Y	**N**	Equal volume mixture of 5 plasmid preps from 012107
pCZ479a	Y	Y	prepared from E10-012107
pCZ479b	Y	Y	prepared from E8-012107
pCZ479c	Y	Y	prepared from E6-012107
pCZ479d	Y	Y	prepared from D8-012107
pCZ479e	Y	Y	prepared from D9-012107
c1-031607	Y	Y	
c2-031607	Y	Y	
c3-031607	Y	Y	
c4-031607	Y	Y	
c5-031607	Y	Y	
c6-031607	Y	Y	
e1-031607	Y	Y	
e2-031607	Y	Y	
e3-031607	Y	Y	
e4-031607	Y	Y	
e5-031607	Y	Y	
e6-031607	Y	Y	
pCZ479	Y	Y	Equal volume mixture of all 12 plasmid preps from 031607

Section 3.4.5 Selection of Stably Transfected Clones

Transfection of the cells was performed in 96 well plates that contained cells which were actively growing and less than 50% confluent. Approximately 24 h after transfection, media containing 1000 µg/mL of G418 was added. G418 was used to select for the plasmid-conferred expression of neomycin resistance gene. Fresh G418-containing media was added every 2-3 days. After most of the cells had died and

detached from the monolayer and signs of small colony formation were visible, which typically took about 3 weeks, the wells were examined and the total number of individual clones of cells were recorded. Only the wells containing one stably transfected cell clone were used from this point forward. The cells in these wells were trypsinized and reseeded back into the same well and allowed to grow to near confluence. The cells were then split and divided among 3 different new plates, one of which was infected with ADV and monitored for GFP expression. Some of the clones were seeded into larger flasks and expanded and frozen for long term storage.

Chapter 4

RESULTS AND DISCUSSION

Section 4.1 Adenovirus Time-Kill Testing

Prior to the commencement of this project, our laboratory had minimal experience working with ADV or with antiviral efficacy testing. Therefore, the first milestone involved gaining proficiency in the basic laboratory techniques required to perform antiviral testing against ADV. The cell line to use had to be chosen, virus stocks needed to be prepared and proficiency in quantifying the infectious particles had to be gained. Once the basic techniques were successfully established the next step was to learn how to perform the existing standard ADV TK assays, PA and CCID50.

Section 4.1.1 Establishment of Experimental Conditions for Plaque Assay

An aliquot of the purchased virus stock was used to prepare the first working viral stock solution designated Ad021505. Several initial attempts to titer Ad021505 failed to yield countable plaques. The first procedure used was modeled after the method currently in use in the laboratory for herpes virus plaque formation. Confluent HeLa or Vero cells were infected with virus in HBSS solution for 60 m followed by the addition of a 1.5% methylcellulose in medium overlay and incubation for 3 days. After the methylcellulose was removed, the cells were stained and fixed with a crystal violet solution prepared in ethanol. No plaques were observed in the dilutions tested (10^{-4} to 10^{-8}) in HeLa and Vero cells using this procedure. In this and all future PA experiments to be described, a negative control well without virus and a positive control well with 100 µL of undiluted

virus stock preparation were utilized. The negative control wells, unless otherwise noted, did not show evidence of CPE while the positive control wells no longer had evidence of any viable cells, e.g. 100% CPE was observed. When the incubation time was increased to 7 days and dilutions as little as 10^{-2} were plated some evidence of CPE on the cells could be distinguished in the 10^{-2} wells and to a lesser extent in the 10^{-3} wells. Increasing the incubation time to 7 days for the HeLa cells required addition of new media at least once during the incubation since the HeLa cells would die rapidly without this step. The CPE was distributed evenly among all of the cells in the well. There were no concentrated areas of CPE or clear zones of cell death so it was not possible to count plaques. The conclusion at the time was that the concentration of the prepared virus stock must have been too low.

In an attempt to achieve a higher viral titer, three new batches of virus stocks were prepared using slightly different methods than the original prepared stock. The FBS concentration in the media was changed from 10% to 2% once the virus was added to the cells. The purpose of this change was to slow the growth of the cells in hopes that this would limit cell growth and sustain the monolayer to allow the virus to form countable plaques. The volume of media added after infection of the cells was decreased so that the resulting virus concentration would be concomitantly increased. In one of the preparations, the viral mixture was centrifuged in order to remove any cell debris. A 60 m virus adsorption step in a minimal volume of buffer solution was added to the protocol as well to increase the likelihood that each virus would be able to infect the cells. When these three new virus stock batches were titered using the existing plaque assay method the results were similar to those using the Ad021505 preparation. In fact, it appeared that

they all had slightly lower virus concentrations. In order to determine if the 60 m virus adsorption step being used in the PA procedure was the optimal length of time, some of the evaluations were repeated using 30 and 90 m also. The results were identical regardless of the time the virus was allowed to adsorb to the cells.

At this point it seemed that one of two things was occurring. Either the virus preparation procedure was flawed, which resulted in very low titers, or the PA procedure was underestimating the true concentration of virus. Virus purchased from ATCC that was used to create the lab batches of virus stocks contained approximately $10^{6.5}$ PFU/mL of ADV. Virus from the ATCC vial was titered alongside the lab virus stock batches. The results showed that the lab batches were actually about 1 log more concentrated than the ATCC sample. The cells infected with the ATCC sample showed minimal CPE in the 10^{-3} well and significant CPE in the 10^{-2} well but none in the wells receiving 10^{-4} and higher dilutions. The lab-prepared batches showed some CPE in the 10^{-4} well and significant CPE in the 10^{-2} and 10^{-3} wells while showing none in the higher dilutions. By comparison to the known ATCC stock virus, it was inferred that the lab virus stock preparations contained about $10^{7.5}$ PFU/mL, but that the plaque assay procedure in use was not allowing all of the virus particles to infect the cells. In addition, there was still the significant problem of non-distinct plaques. CPE was generalized across the cells in the whole well. It appeared that the virus was able to move through the methylcellulose and was not localized to enable plaque formation. Furthermore, the HeLa cells were not particularly robust and required diligent maintenance to be kept alive for the 7 days required for CPE to form.

Numerous variations of the method were explored to try and overcome these initial shortcomings, most of which were shown to have no effect on the results. Media 199 was compared to DMEM media. Different FBS concentrations, 2%, 5%, and 10%, were tested. The methylcellulose was removed and compared to the use of just the liquid media by itself. The effect of leaving the HBSS and virus mixture in the plate after virus adsorption was compared to the result when it was removed. Use of cells at levels of confluence from 10% to 100% were compared. Results obtained using all of these variations were indistinguishable and unsatisfactory.

Clear countable plaques were first observed when agarose was used instead of methylcellulose in the overlay and when the incubation time was increased to 11 days. When agarose was used as the overlay distinct plaques could be counted as early as 5 days after infection. The titer of the original preparation of virus stock, Ad021505, was determined by this method to be 2×10^7 PFU/mL. Distinct plaques did eventually form even with the methylcellulose overlay, but this required 10-11 days and they weren't as easy to count and were not as clearly defined as they were in the agarose-containing plates. All further PA experiments were performed using the agarose overlay.

Section 4.1.2 Effect of Several Variables on the Observed Virus Titer

Even though the PA method at this point produced countable plaques that could be used to quantify virus, the assay was still quite cumbersome to perform due to 1) the difficulty in maintaining the HeLa cells for the length of time needed to observe plaques, 2) the plaques were undefined and not very easy to count, and 3) the virus titer obtained was still not high enough to be used in TK testing. Further optimization was aimed at

increasing the ease of performance of the test and increasing the virus concentration. In addition, the accuracy and precision of the results obtained so far were not known.

Additional virus stock inoculum preparation procedures were examined. First, it was hypothesized that some of the virus produced may have been discarded in the pellet after centrifugation due to incomplete lysis of the cells during the freeze-thaw cycles. In order to determine if the virus was in the cells, trials were conducted in which the pellet was kept while the supernatant was discarded. Also, the resulting viral suspensions were passed through syringe needles of 20, 26 and 30 gauges in order to shear cells and release more virus from the cells. Vigorous vortexing of the viral inoculums for 5 minutes was tested as well. None of these variations in inoculum preparation procedure significantly changed the resulting viral titer.

Several additional procedure modifications were identified and tested based on a review of published procedures [23,24]. Of the following modifications that were examined, none had an effect on the observed titer. These included: addition of L-glutamine to the media, washing the cells with serum-free media, addition of $MgCl_2$ to the media, using serum-free media in place of HBSS, using methylcellulose made with DMEM instead of 199 media, and using various concentrations of agarose in the overlay.

Only two factors significantly changed the observed titer result, the length of incubation time and the use of a different cell line. During a previous experiment, the concentration of the Ad021505 virus stock was determined to be 1.2×10^7 PFU/mL after 5 days of incubation. However, in one of the later experiments in which the plates were incubated for 11 days, the observed titer of the same virus stock was determined to be 3.0×10^8 PFU/mL. In addition, since most literature references and the ASTM E1052

recommend using A549 cells when working with ADV, these cells were tested. Once A549 cells were utilized instead of HeLa cells, the assay became much easier to perform. The plaques formed in the A549 cells were very distinct with clear edges and stained a much darker purple color with crystal violet. This allowed for more rapid and accurate counting of plaques relative to the prior work using HeLa cells. The A549 cells remained alive and relatively healthy with minimal maintenance and did not require additional fresh media for up to 2 weeks. In addition, the titers obtained using the A549 cells were higher than those obtained with the HeLa cells. In addition to the heartiness of the A549 cells, it appeared that ADV infects the A549 cells more efficiently than HeLa cells. The results from the experiments illustrating the effects of cell type and incubation time are shown in Figure 4.1.2. The standard PA procedure, therefore, was established as one that uses an agarose overlay, A549 cells, and one which allows 7-8 days for incubation.

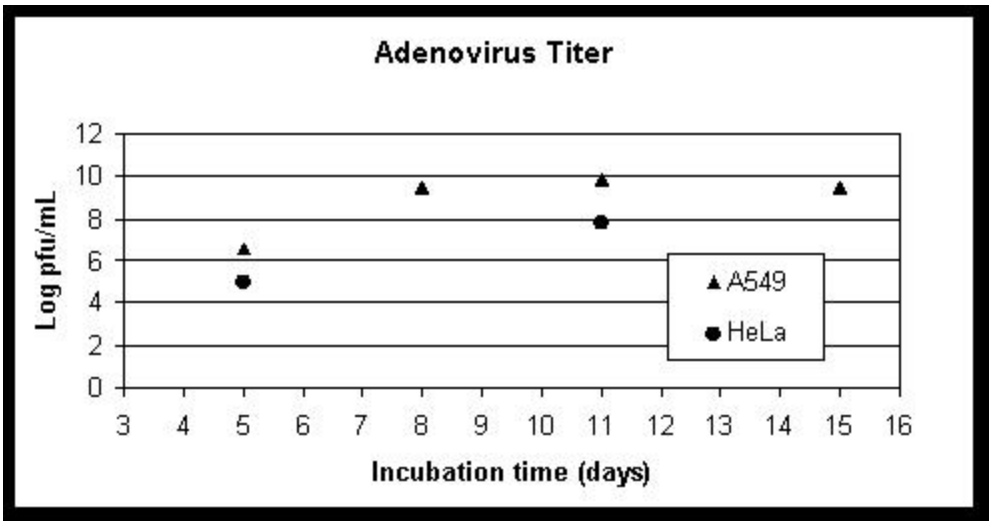


Figure 4.1.2 ADV Titer vs. Incubation Time and Cell Type

Section 4.1.3 ADV TK Tests with PA and CCID50

After the standard PA procedure was established for determining ADV PFU/mL, the next step was to develop the traditional anti-ADV TK assays. Several rounds of testing using the CCID50 and PA protocols were performed to determine the TK results for various concentrations of ethanol and bleach (hypochlorite) as well as PURELL and other ethanol-based formulations. Many of the test formulations had been evaluated previously by a contract laboratory, ATS Labs, which utilizes the CCID50 protocol. After performing multiple rounds of testing using both traditional methods, the PA method quickly stood out as the preferred method.

The CCID50 method was relatively difficult and time-consuming to perform. The CCID50 method required subjective examination of the cells and grading of their % CPE as compared to simply counting well-defined, stained plaques in the PA method. In addition, the need to observe the cells for CPE on a certain day was not as convenient as the PA which could be counted anytime after staining. The PA method also had the added benefit of producing plates that could be retained as permanent records of the experiments. Lastly, the need to view each of many wells under the microscope in the CCID50 method was less ergonometric since it was more comfortable to count the plaques in the PA method on a light box than it was to view cells under the microscope. For these reasons, only the PA method was performed in future experiments except when it was necessary to demonstrate the equivalence of the PA method to the CCID50 method.

The purpose of the first few rounds of testing was primarily to become familiar with the methods and to begin to determine their precision and accuracy. Accuracy was defined in terms of how close the results matched those obtained by ATS Labs.

Whenever possible, samples that were tested multiple times at ATS Labs were used since even their results showed considerable variability between trials for some test samples. As Table 4.1.3 illustrates, early trials of both methods indicated some precision but little accuracy. Our internal LRs for the PA and CCID50 methods generally were similar to each other, but both were considerably lower than those obtained by ATS Labs for the same or very similar samples.

Table 4.1.3 Comparison of Internal to ATS Results

	Log Reductions		
	ATS Labs	Internal	
Test Sample	CCID50	PA	CCID50
78% Ethanol	>4.25, >5.00, 4.00, >6.00, >5.00	1.2	0.5, 0.0
118 ppm NaOCl ~100 ppm NaOCl 87 ppm NaOCl	not tested >6.00, >6.75 not tested	2.0 not tested 0.1	2.0 not tested not tested
PURELL	2.50, 3.00	not tested	0.0
modified PURELL-517	1.50, >6.00	3.5	not tested
modified PURELL-2029	not tested	1.2	1.0, 1.0
modified PURELL-2188	>5.00	2.8, 2.0	not tested

Section 4.2 Plaque Assay Optimization

The following experiments were performed to determine why our LRs were lower than those obtained by ATS. A description of the procedures used by ATS was obtained by personal observation of the method being performed at their laboratory as well as through telephone conversations and review of their written procedures. A thorough comparison of our protocol to the ATS protocol generated several theories. As the following sections will describe, the conclusion obtained after systematically ruling out many incorrect theories was that differences in the temperature of the test sample when it was exposed to the virus was the major issue causing our test results to be lower.

Furthermore, our results were also being affected by differences in viral inoculum concentration and the viral inoculum preparation procedures.

Section 4.2.1 Observed Log Reductions are Affected by Initial Viral Titer Concentration

The LR for one test sample was evaluated using initial challenge titers between 7 and 10 log PFU/mL in order to determine the relationship between the amount of virus in the TK test and the observed LR. At higher challenge inoculums there is more virus in the sample to be inactivated and this can result in reduced LRs since the ratio of sample to virus is decreased and the amount of active ingredient required to inactivate the greater number of virus should also be higher. For example, to achieve a 2 LR following treatment of a sample with 8 logs of virus, the treatment would have to inactivate a total of 99,000,000 PFU (100,000,000 – x = 1,000,000) whereas for a sample with an initial titer of 9 logs, the number of PFU to be inactivated to achieve the same 2 LR increases to 990,000,000 PFU (1,000,000,000 – x = 10,000,000). Some active ingredients get used up when they interact with the viral inoculum and results for these agents would be inherently more susceptible to this effect. For these reasons it was initially hypothesized that by lowering the initial viral titer, LRs closer to those observed at ATS could be achieved.

Viral inoculums between 7 and 10 logs were tested. Figure 4.2.1 shows that the observed LR did decrease as the amount of virus increased. The decrease in LR was only about 0.5 logs for each 10 fold increase in virus in the initial challenge, at least for the sample that was tested in this experiment. Since the variation between the titer

concentrations used at ATS and those used in our earlier experiments was only about 1 log, these differences alone were not great enough to account for the magnitude of difference between our results and the ATS results. The data in Figure 4.2.1 also reveals that the assay is very reproducible and the results observed for this test sample were very precise since the results shown were obtained on 4 different test dates which spanned 3 months.

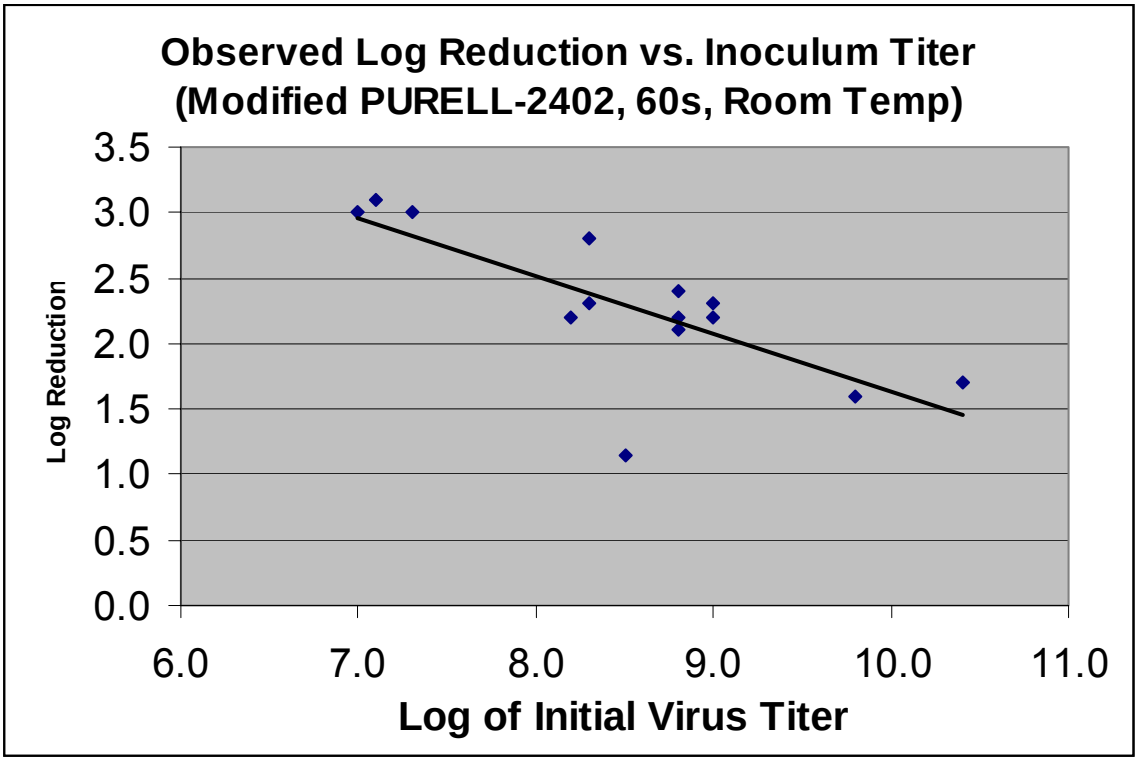


Figure 4.2.1 Observed Log Reduction vs. Inoculum Titer

Section 4.2.2 The Effect of Inoculum Preparation Procedure on Log Reductions

A major difference between the ATS method and our method was the procedure used to produce the viral stocks that were used to test the samples. First, ATS produced virus in A549 cells, but the virus stock we were using was produced from HeLa cells. We

hypothesized that perhaps the virus could be slightly different and more resistant to biocides because it was produced from the different cells. In addition, ATS always used cells <50 % confluent for infection when producing ADV stocks, whereas we were using cells that were ~90% confluent. They also added a centrifugation step to remove the cell debris from the mixture after the freeze-thaw steps whereas our stock still had the cell debris in it. It seemed highly probable that the additional cellular debris present in our virus stocks could have decreased the ability of the test products to inactivate the virus and thus resulted in lower LRs. Furthermore, it was discovered that ATS used media containing only 1% FBS vs. the 2% FBS that we were using, which could also interfere with virus inactivation. Furthermore ATS began the first freeze cycle 2-3 days after the cells were infected, before any visible CPE was obvious. In contrast, we had waited 4-5 days before starting the first freeze cycle. Lastly, ATS infected their cells with a MOI approximately 10X greater than we were using during the initial infection (100 vs. 10).

A new stock of ADV (Ad092405A) was prepared using A549 cells using the procedure described by ATS and compared to our original stock of ADV that had been used for all prior testing (Ad021505). In addition, cell debris in the original ADV stock, Ad021505, was removed by centrifuging and discarding the pellet in the preparation designated Ad092405B. All 3 viral preparations were used to evaluate the antiviral efficacy after a 60 s incubation at room temperature (RT) of a solution of 226 ppm NaOCl or an ethanol-containing test formulation, modified PURELL-2402. It was quite surprising and disappointing that the results were nearly identical between the trials with Ad021905 and Ad092405A. The 226 ppm NaOCl sample showed final LRs of >4.8 and >5.4 while the modified-PURELL-2402 resulted in log reductions of 1.6 and 1.7. No

results were obtained using the Ad092405B preparation because the plate used for this trial had viral contamination of the cells in all the wells, including the negative control wells.

In a later experiment, however, a significant change in result occurred when the original Ad021505 virus preparation was used and compared to the new Ad092405A. In this particular experiment PA and CCID50 methods were both employed and duplicate trials were conducted using each method with a 60 s incubation at RT. As shown in Table 4.2.2, the observed LRs for trials of a 78% ethanol solution were significantly higher using the virus stock preparation prepared using the ATS method. When a different test sample, modified-PURELL-3051, was tested there was only a slight increase in LRs. Overall the lesson learned was that the virus inoculum preparation procedure can significantly affect the observed results, but the extent of the effect is not the same for all types of samples.

Table 4.2.2 Log Reductions using Different Virus Stock Preparations

	CCID50		PA	
Log Reductions from two replicates	GOJO viral stock (Ad021505)	ATS virus stock method (Ad092405A)	GOJO viral stock (Ad021505)	ATS virus stock method (Ad092405A)
78% Ethanol	2.0, 2.0	>3.0, >3.0	2.9, 3.3	>4.1, >4.1
modified PURELL-3051	1.0, 1.0	1.0, 2.0	1.3, 1.3	1.7, 1.7

Section 4.2.3 Cell Confluence and Dilution Solutions

ATS had used cultures that were <50% confluent for the ADV test, while our tests had used cultures that were about 80-90% confluent. The next experiment was designed to evaluate the effect of the variable confluence of the cells on observed LRs. The ADV

TK test was performed using the AD092405A virus preparation. The resulting dilutions were plated onto cells that were completely (100%) confluent (36 h post plating) and on cells that were only 21h old and barely (80%) confluent. Modified-PURELL-2402 was tested again and resulted in a 2.3 LR on the older cells and a 2.1 LR on the younger cells. As part of the viral inoculum concentration experiments discussed earlier in section 4.2.1, 1/10 and 1/100 dilutions of the AD092405A were also tested against both the old and young cells. Again the results were indistinguishable between the plates: 2.3 LR on old cells vs. 2.2 LR on the younger cells for the 1/10 dilution and 3.0 LR on the older cells vs. 3.1 on the younger cells on the 1/100 dilution. Confluence levels of the cells between 80% - 100% yielded similar results. Ideally cells with confluence levels <50% should have been used for this experiment to rule out the effect of cell density on observed LR, but the cells were inadvertently seeded too densely. Based on these observations it seemed improbable that cell density was a major factor affecting LR results.

The ATS method also used different solutions in the dilution tubes. We had been using a neutralizing solution in the first dilution tube that consisted of 50% FBS and 0.5% sodium thiosulfate and EBSS in all of the remaining tubes. ATS uses 100% FBS in the first tube followed by dilution into media containing 1% FBS for all of the remaining tubes. The LRs were compared for 16 tests, 8 using the ATS dilution tubes and 8 using our standard dilution solutions.One of the 8 pairs of runs resulted in a 0.4 log increase for the ATS method vs. our method, while the 7 other runs resulted in identical LR values between trials (5 of which were identical because both showed total inactivation up to the limit of the test). Even though this test showed that the solutions in the dilution tubes

were unlikely to alter the results, subsequent assays were performed using the ATS recommended dilution solutions.

Section 4.2.4 Variability in the LRs for One Formulation

The next experiment was designed to test the idea that the control sample 2402 that we had used in numerous previous tests may have changed or degraded rendering it less efficacious than it may have been when it was initially tested at ATS. We hypothesized that the ethanol was evaporating after repeatedly opening and closing the sample storage container. Additional batches of the same formulation were obtained including 2 that were older than this one (517, 2188) and one that was newer, 3051. The observed LRs for the 4 samples when tested for their efficacy at 60 s at RT from oldest to newest were: 3.5, 2.0, 2.4, 1.3. The conclusion was that although there was no direct correlation between the age of the sample and efficacy, the rather large spread of LR, spanning 2.2 logs, revealed that some inherent variability between batches of the sample may exist and therefore it is possible that our reduced LRs were the result of a change in the test sample 2402 due to its storage and handling conditions. Therefore it was decided that this sample would not be used in future testing since we did not have the luxury to have it retested by ATS.

Section 4.2.5 Comparison of Results between the PA and CCID Methods

Since our results using the PA procedure were still significantly different than those obtained by ATS, who had used CCID50 methodology, we decided that additional testing comparing the two methods was needed even though previous results had yielded

similar results with both methods. A total of 16 different trials were conducted and the resulting dilutions were quantified using both the PA and the CCID50 methods. Since the results from these CCID50 trials represent one individual trial each, not an average of quadruplicate plates as is customary, the results are only a rough estimate with an assumed variability of at least +/- 1 log. As Table 4.2.5 illustrates, the results between the two methods were very similar. This confirmed that the major reason for the differences in observed LRs was not due to our use of the PA method vs. ATS's use of the CCID50 method.

Table 4.2.5 Comparison of PA and CCID50

Sample	Temp	Log Reduction	
		PA	CCID50
2224	RT	3.3	2
		>4.1	>3
		2.9	2
		>4.1	>3
	37C	>3.8	>3
		>4.1	>3
		>3.8	>3
		>4.1	>3
3051	RT	1.3	1
		1.7	1
		1.3	1
		1.7	2
	37C	>3.8	>3
		>4.1	>3
		>3.8	>3
		>4.1	>3

Section 4.2.6 Effect of Temperature of the Test Sample on Log Reductions

The virus was exposed to test formulations that were at RT during most of the prior experiments. RT varied from about 20 to 25 ºC. Some of the results from ATS were conducted at 25 ºC while some were conducted at 33 ºC. 33 ºC was chosen because it is

the temperature of the surface of skin and the products being tested were designed to be efficacious on the skin. Preliminary comparisons between results obtained at ATS using the two different temperatures for similar test formulations indicated that there wasn't a great effect on the observed LRs. Based on that observation the exact temperature used in testing was not precisely controlled in earlier testing. After a visit to ATS it was noted that the technicians were equilibrating the test samples and the virus inoculum in a water bath up until the moment of testing. The effect of the temperature of the test sample on the observed LRs was investigated.

In the first experiment, two samples, 2188 and 2402, were evaluated at RT and at 33 ºC. The result was that the LR for sample 2188 increased from 2.0 at RT to 2.8 at 33 ºC. Sample 2402 changed more drastically, going from 2.4 to 4.5 with the increase in temperature. Next a larger test was performed using two additional samples 2224 (78% ethanol in water) and another ethanol-based prototype 3051. During the course of the experiment several variables were tested, but each condition was tested at RT and 37 ºC. The results as shown in Table 4.2.5 clearly show the increase in LR that accompanies an increase in temperature during the incubations. The new LRs that were obtained were more similar to those obtained by ATS, therefore further optimization of the traditional method protocol was stopped and careful control of the test formula sample was incorporated into all future tests.

Section 4.3 Evaluation of ADV-GFP Method

After establishing the capability to perform ADV TK testing using the PA and CCID50 traditional methods we were now able to turn our attention to the project goal

which was to evaluate new improved methods. We began by conducting TK testing using the recombinant ADV-GFP recombinant virus and utilizing fluorescence measurements to quantify the virus. Quantification of GFP expression was shown to result in a similar titer as results obtained using a PA procedure, however, in these previous experiments ADV type 5 was used for both the assays and the result of kill tests were not compared [25]. In our experiments we needed to know if the ADV-GFP type 5 recombinant virus would be equally susceptible to kill as the type 2 *wt* virus.

Several rounds of TK testing were performed using the ADV-GFP virus and test formulations of various concentrations of bleach and ethanol until a dose-response curve was obtained for each active concentration. Additionally, these tests were compared to the PA procedure. The assay was used to evaluate incubations at multiple temperatures, as well. As Figure 4.3a illustrates, the results for ethanol solutions obtained by PA using *wt* ADV with A549 cells were nearly identical to those obtained using ADV-GFP with HEK293 cells and utilizing GFP expression to gather results. Similar testing was conducted using various concentrations of bleach samples. Figure 4.3b shows the results of these experiments. Again, the results obtained for the two assays were nearly identical. This indicated that ADV-GFP could be used in place of the *wt* ADV and results could be obtained in only about 2 days since it wasn't necessary to wait for evidence of CPE.

Section 4.4 Creation of Cells that Conditionally Express GFP

The ultimate goal of the next phase of experiments was to create a cell line that fluoresces upon infection with ADV and could be used in ADV TK testing. The strategy for creation of this new cell line was to incorporate a genetic construct which has the

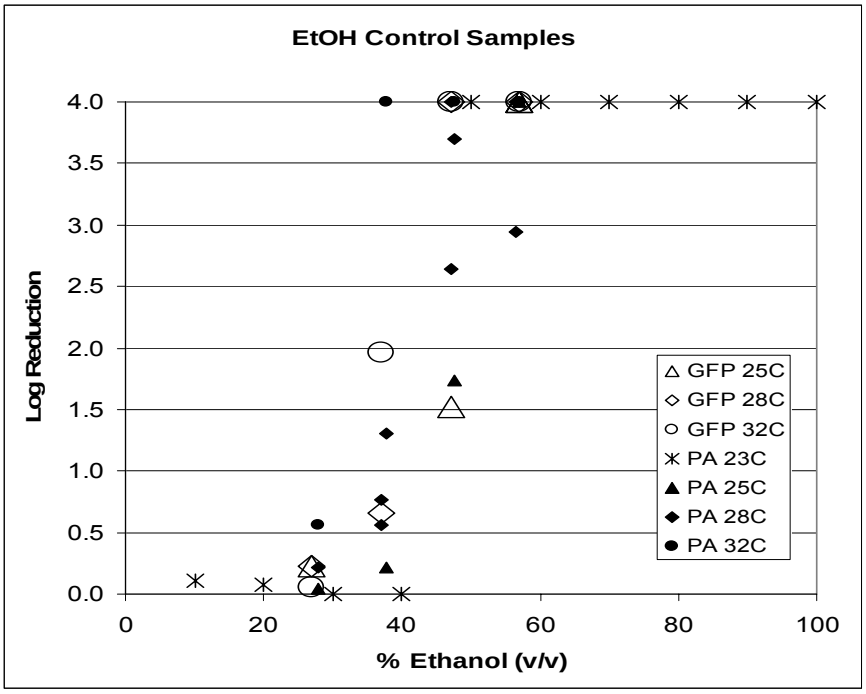


Figure 4.3a ADV-GFP vs. PA for Ethanol

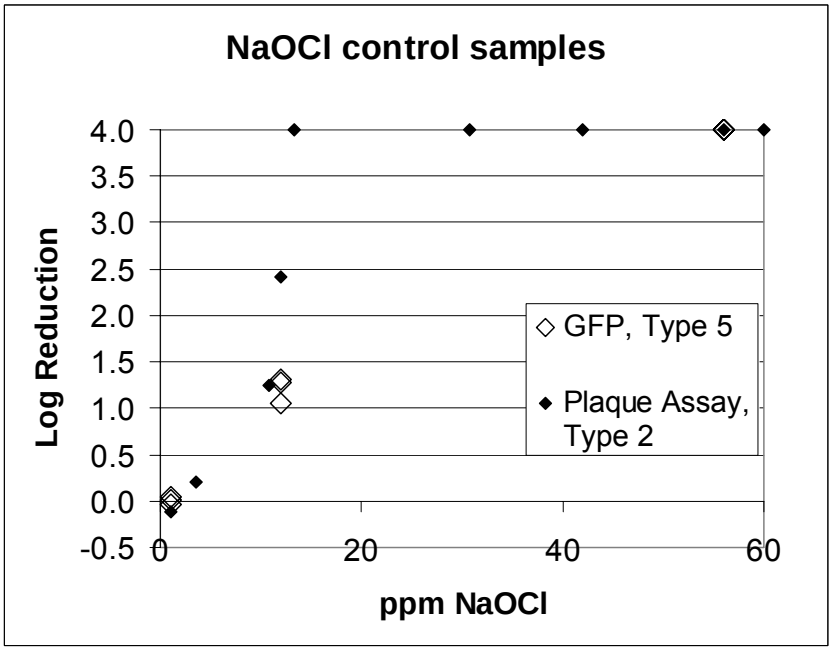


Figure 4.3b ADV-GFP vs. PA for NaOCl

promoter from an early ADV gene upstream from the GFP gene into the cell's chromosomes.

ADV has 4 early genes, E1, E2, E3, and E4. We chose to utilize the E2 early gene for several reasons. E1 is automatically made early in ADV infection and is under host cell control so its promoter would not be specifically activated by ADV infection. E3 was not a good choice because it is not essential *in vitro* since it functions to assist the host at evading the immune system and therefore it might not be expressed consistently [26]. Both E2 and E4 were acceptable choices since their full sequences are known [22,27], both are essential, both are only expressed after ADV infection, and their expression is turned on by E1a, which is produced soon after ADV infection. Thus, expression of the E2 or E4 genes can be used as indicators of ADV infection. E4 makes proteins that help increase viral gene expression and decrease cellular gene expression. E4 was shown to be expressed 20X more in the presence of E1a, but that it is expressed at a low level even in the absence of E1a [27]. The E2 gene promoter was shown to contain an enhancer that works at nearly any distance from the E2 gene and in any orientation [22,28]. Based on this information the E2 gene promoter was selected to be the promoter that we would use to regulate expression of GFP in the promoterless vector. We chose to use A549 cells as the host cells because they are hardy and can be infected by ADV. A promoterless vector containing the GFP gene, phrGFP, was obtained from Strategene and all procedures followed the recommended protocols in the accompanying Stratagene manual. Figure 4.4 summarizes the overall strategy employed in the process of creating the new cell line and verifying the success of each step.

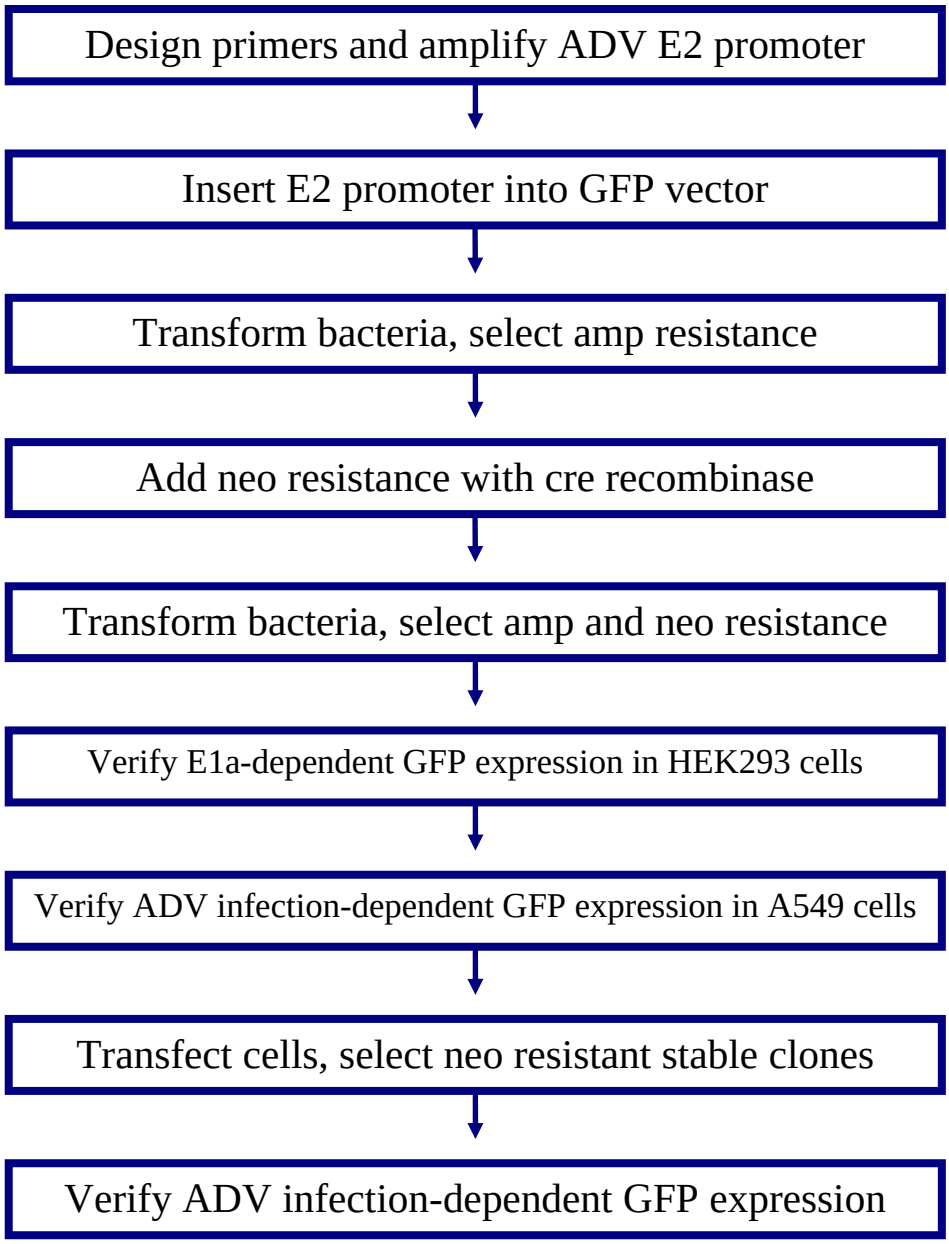


Figure 4.4 Strategy for Development of the New Cell Line

Section 4.4.1 Amplified Adenovirus E2 Promoter

PCR amplification of the E2 promoter sequence was conducted using target DNA

from cells infected with *wt* ADV and primers that flank the E2 promoter region. Successful amplification of the E2 promoter sequence was verified using gel electrophoresis, as illustrated in Figure 4.4.1. A DNA band appeared between 100 and

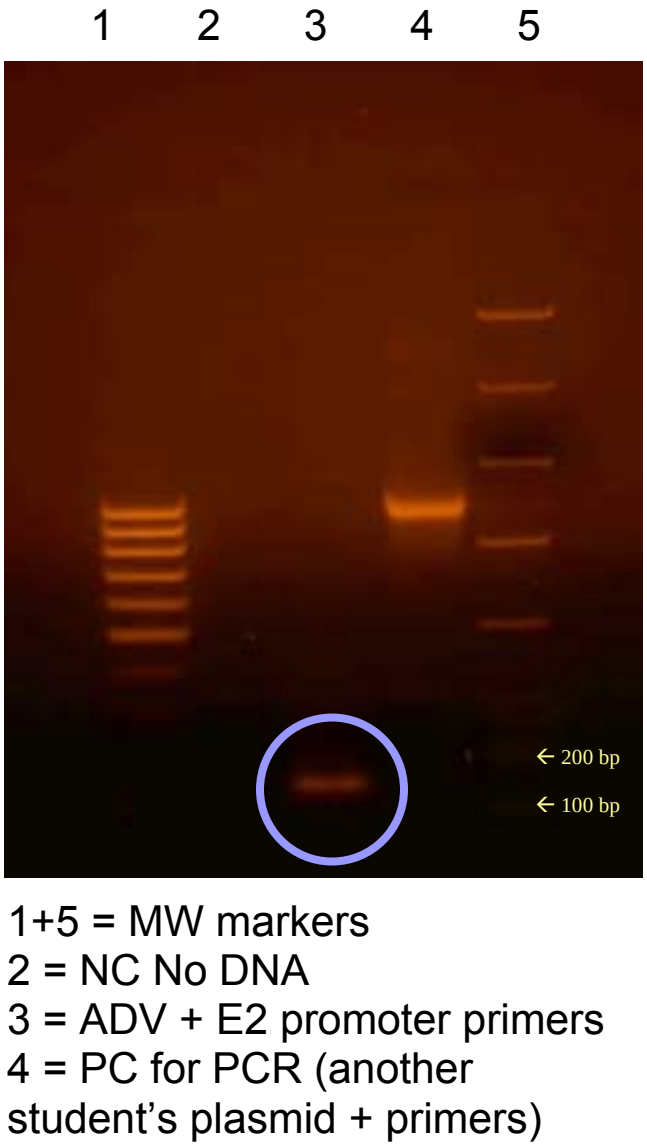


Figure 4.4.1 PCR Amplification of E2 Promoter

150 bp in the test lane. In contrast, the negative control lane without any DNA did not show a band. Another control sample used a different set of primers and target DNA from

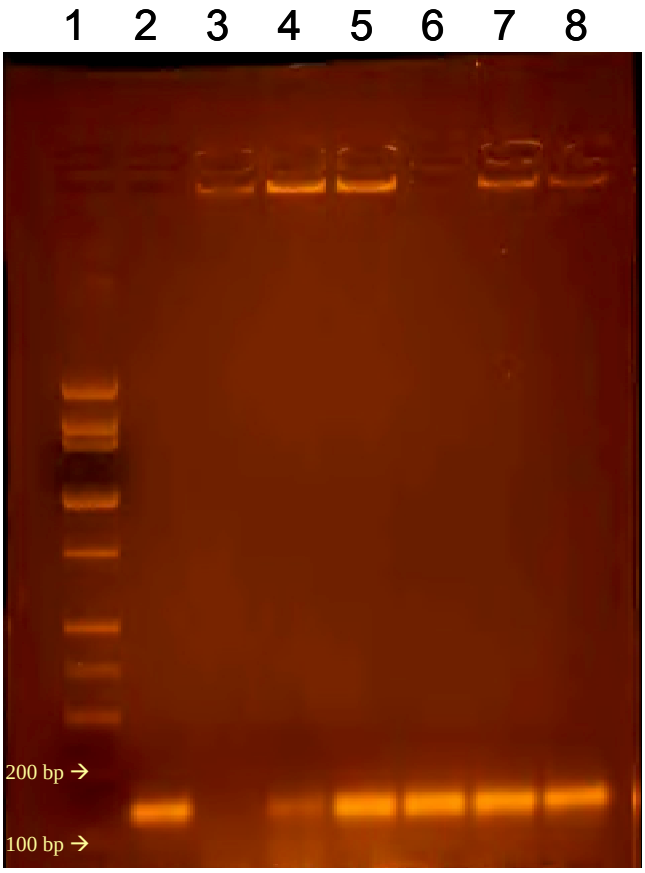
another student's project. A band appeared at the expected size which indicated that the polymerase, PCR reagents and instrument were all functional.

Section 4.4.2 Preparation of Plasmids

After restriction enzyme digestion and ligation of the E2 promoter fragment into the Stratagene vector and bacterial transformation with the plasmid, presence of the E2 promoter sequence was verified by colony PCR using the E2 promoter primers. As illustrated in Figure 4.4.2, the colonies were shown to contain the sequence as was evident by the appearance of bands between 100 – 150 bp. To complete the construction of the final plasmid, neomycin resistance was added using the cre/lox system and the final construct was transformed into *E. coli*. Selected colonies exhibiting ampicillin and kanamycin resistance were shown to contain the E2 promoter sequence by colony PCR.

Section 4.4.3 Verified E1a-Dependent GFP Expression

The next step in the development of the E1a dependent GFP plasmid was to test expression of the GFP gene in response to activation of the E2 promoter by the E1a protein. HEK293 cells, which constitutively express the E1 gene from ADV and therefore continuously produce E1a protein, were transiently transfected with many of the prepared plasmids and the expression of GFP was monitored. These experiments verified that the E2 promoter was able to turn on the GFP gene in the presence of E1a protein in the newly created plasmid constructs. In the first experiment, 5 plasmids were transiently transfected into HEK293 cells and after 2 days, the number of fluorescent cells for each



1 = MW marker

2 = PC Adenovirus + E2 promoter primers

3 = NC No DNA

4-8 = Different transformed E. coli colonies

Figure 4.4.2 Colony PCR to Verify Promoter Presence

of the plasmids was counted. As shown in Table 4.4.3a and Figure 4.4.3, cells transfected with the plasmids containing the E2 promoter showed significantly more expression of GFP than those transfected with the phrGFP plasmid without the E2 promoter. Only one cell in the well transfected with phrGFP expressed GFP while the 4 other plasmids tested

showed between 26 and >300 fluorescent cells. In this experiment, the DNA plasmid mixture for the plasmids with added NEO resistance (pCZ479e and pCZ479c) that were used for transfection had inadvertently not undergone a DNA purification step.

Table 4.4.3a Transient Transfection of HEK293 Cells

	Negative Control	**phrGFP**	**D8-012107**	**E10-012107**	**pCZ479e**	**pCZ479c**
E2 promoter	n/a	No	Yes	Yes	Yes	Yes
NEO resistance	n/a	No	No	No	Yes	Yes
Green cells	0	1	56	>300	42	26

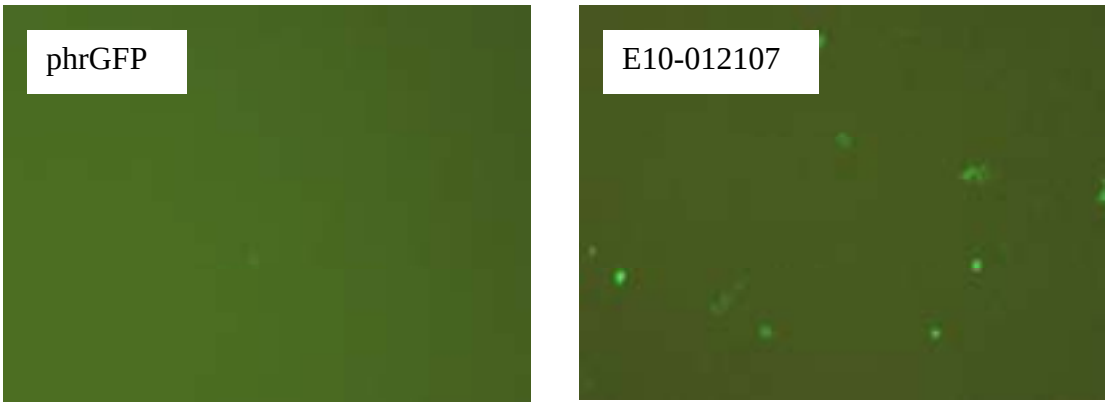


Figure 4.4.3 Fluorescence Images of Transient Transfection of HEK293 Cells with plasmid

The results from another experiment using all of the created plasmids yielded similar results (see Table 4.4.3b). Most of the plasmids containing the E2 promoter controlled GFP gene showed 300-500 green cells in the well. In this experiment the plasmids with the neo resistance gene performed as well as those without it. In addition, wells transfected with the phrGFP plasmid or untransfected wells showed no green cells.

This demonstrated that the plasmids were functional. The presence of E1a protein was able to turn on GFP expression in the construct with the E2 promoter but not in the plasmids without the ADV promoter. This verified that the GFP gene in the constructs were under the control of the E2 promoter.

Table 4.4.3b 2nd Transient Transfection of HEK293 Cells

Plasmid	Green Cells
D8-012107	Approx 300-500
D9-012107	Approx 300-500
E6-012107	Approx 300-500
E8-012107	Approx 300-500
E10-012107	Approx 300-500
E2promMIX	Approx 300-500
c1-031607	Approx 300-500
c2-031607	Approx 300-500
c3-031607	Approx 300-500
c4-031607	Approx 300-500
c5-031607	0
c6-031607	Approx 300-500
e1-031607	Approx 300-500
e2-031607	Approx 300-500
e3-031607	Approx 300-500
e4-031607	Approx 300-500
e5-031607	Approx 300-500
e6-031607	Approx 300-500
pCZ479	Approx 300-500
phrGFP	0
NC- none	0
NC- none	0
NC- none	0
NC- none	0

Section 4.4.4 Verification of ADV Infection Dependent Expression of GFP in Transiently Transfected A549 Cells

The purpose of the next round of experiments was to determine if GFP would be expressed at observable levels in ADV infected A549 cells that were transiently transfected with the plasmid constructs. Prior experiments had shown that the E2 promoter in the plasmid was governing the expression of the GFP and being turned on by E1a protein, but it was not yet known if the amount and location of E1a that is produced in an ADV infected cell would be sufficient to induce the GFP expression. Also, this set of experiments was used to optimize the transfection protocol for A549 cells. Several different transfection conditions were tested in order to determine the optimal conditions to achieve the highest possible transfection efficiency, but the result was that none of the transfection conditions gave significantly different results. The transfection efficiencies observed were between 0.2% and 2.5%. Table 4.4.4 contains details of the experimental design and results and Figure 4.4.4 contains images from two representative wells.

Overall, the results of the experiments outlined in Table 4.4.4 were very promising and can be considered proof of principle that the E2 promoter controlled GFP can monitor ADV infection and that a cell line bearing this plasmid construct should do the same. A total of 22 trials involved cells that were not infected with ADV, 16 of which were transfected with the final plasmid mixture, 2 with plasmids that did not get the neomycin resistance gene, 2 with the original empty Stratagene vector, and 2 which were not transfected with any DNA. No expression of GFP was observed in any of these trials. This indicates that the GFP gene in the construct is not leaky and the E2 promoter is not turned on in uninfected cells. Cells in two trials were not transfected with any DNA but were infected with ADV, they also had no green cells, which showed that the cells alone

did not contain the GFP gene or any another fluorescent protein that ADV infection would turn on.

GFP expression was compared in cells infected with ADV after transient transfection with either the final plasmid mixture, plasmids lacking the neomycin resistance gene, or the original Stratagene vector. When observed two days after infection, the wells transfected with phrGFP had an average of 22 green cells/well, the E2promMIX transfected wells had an average of 168 green cells/well, and the pCZ479 transfected cells had an average of 67 green cells/well. Expression of GFP was indicated by bright, green, easily observed cells, as illustrated in Figure 4.4.4. The lower numbers of green cells observed in the pCZ479 as compared to the pE2promMIX transfected cells may be due to a loss in the number of effective plasmids in the pCZ479 plasmids that may have occurred during the addition of neo resistance to the plasmid. Based on these results it became apparent that ADV infection alone can induce the phrGFP plasmid to produce GFP even though it does not have a promoter. Since this expression of GFP without the promoter occurred in infected cells but not in uninfected cells, it must be related to the ADV infection. Perhaps the added stress on the cell related to ADV infection as well as the increased activity in the cell resulting from the ADV hijacking the cellular processes to create more ADV resulted in increased gene expression and expression of the GFP. The fact that no green cells were observed in the wells transfected with the phrGFP without the presence of ADV infection was the critical control indicative of the functional success of the final cell line.

Table 4.4.4 ADV Infecction of A549 Cells Transiently Transfected with Plasmid

Plasmid	# Green Cells ADV Infected	# Green Cells NO Infection
pFINAL	35, 42, 103, 92, 67, 78, 85, 81, 64, 35, 50, 53, 39, 50, 46, 18, 64, 195 (AVG = 67)	0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0
E2promMIX	106, 250 (AVG = 178)	0, 0
phrGFP	25, 18 (AVG = 22)	0, 0
none	0, 0	0, 0

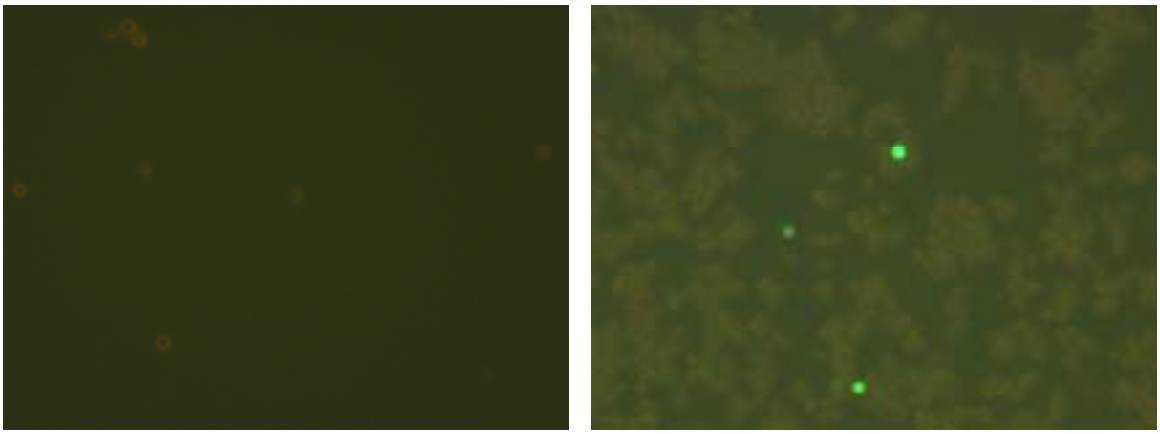


Figure 4.4.4 A549 Cells Transiently Transfected with pCZ479 plasmid. Left = no infection, Right = with ADV infection

Section 4.4.5 Stable Transfection of A549 Cells

At this point we had shown that the pCZ479 plasmid construct contained a functional copy of the GFP gene and the ADV E2 promoter sequence and that the presence of this plasmid in transfected cells resulted in the GFP gene being specifically turned on by ADV infection presumably as a result of E1a protein activation of the E2 promoter. The next step was to use this construct to create a cell line which would have the same properties previously demonstrated by the transiently transfected cells and then finally to use these cells in ADV TK testing. As the following discussion will explain,

stably transfected cells containing the plasmid were created, however, none of the clones that were created demonstrated the desired ADV infection dependent GFP expression.

In order to achieve stable incorporation of the plasmid into the A549 genome, we transfected the cells using the same procedure we used in previous experiments, but then the next day we selected for cells that had incorporated the neomycin resistance gene from the plasmid into their genome. The neomycin resistance gene also confers resistance to the drug G418 which is a drug with a similar mechanism of action to neomycin.

Before selection of stably transfected cells could be conducted, it was necessary to determine the effect of the G418 on the cells to determine the optimal concentration to use for selection. Cultures of nearly confluent A459 cells and HEK293 cells were exposed to the following concentrations of G418: 0 µg/mL, 25 µg/mL, 125 µg/mL, 250 µg/mL, 375 µg/mL, 500 µg/mL, 750 µg/mL, and 1000 µg/mL. The media in the cells was changed and fresh drug added every 2-3 days. Cell death started to occur after about 10 days. In the HEK293 cells some cells were dead in the wells containing at least 125 µg/mL of G418 and all of the cells were dead in wells >375 µg/mL. The A549 cells were slightly more resistant to the G418 drug and signs of cell death began in the well with 250 µg/mL and total death of all the cells occurred in the wells with >750 µg/mL.

In another experiment, the effect of different G418 concentrations on A549 and HEK293 cell survival was examined on frequently passaged growing cells. Cells were trypsinized and diluted to 1:3 or 1:4 their original concentration every 2-3 days and given fresh media with fresh G418. G418 concentrations of 0 µg/mL, 250 µg/mL, 500 µg/mL, and 1000 µg/mL were tested. Both cell lines performed similarly in this experiment. After about two weeks and 4-5 passages, only the flasks containing 1000 µg/mL G418

had no viable cells. Flasks with the 500 µg/mL G418 had about 1/3 as many viable cells as the control flask without G418 while the 750 µg/mL flask contained about 1/10 as many viable cells. Both A549 and HEK293 cells required higher doses of the drug to initiate total cell death when they were being passaged vs. when the media was merely being changed on confluent cells. These experiments indicated that 1000 µg/mL G418 should be used in the upcoming selection of E2pro-GFP permanently transformed cells.

Transfection was performed on several of the wells within 12 well plates. Control wells for comparison received no transfection or transfections of phrGFP or pE2promMIX. Approximately 24 hours after transfection with the plasmids, the media in the wells was replaced with G418-containing media. For 3 weeks the media was changed with fresh G418-containing media. After about 10-14 days, some of the cells started to die. At about 3 weeks, most of the cells had died and the cells that survived were beginning to multiply and create colonies of cells. At this point the cells were trypsinized and diluted in media such that the cell density was approximately 1 cell per 100 µL. The cells were plated into 96 well plates, 100 µL per well. A total of eight 96 well plates were seeded in this way. Every 2-3 days the media was changed using fresh G418-containing media and observed weekly.

After about 1 week of incubation, colonies were visible and those wells containing individual clones were identified and labeled. Approximately 200 of the wells contained individual clones. After about 3 more weeks most of the wells containing individual clones of cells had produced colonies that occupied at least half of the area of the well. At this time each well containing an individual clone was passed and divided

equally among 3 individual wells in new 96 well plates and grown for an additional 1-2 weeks until nearly confluent.

One batch of all of the clones was infected with ADV and observed for GFP expression 2 days after infection. Some general non-specific faint fluorescence was observed in more than half of the clones. Compared to the GFP expression observed using the ADV-GFP virus or to that observed in the transient transfection experiments, this fluorescence was fainter, required greater magnification to visualize and was localized in sections of the cell, sometimes inside, sometimes around the edge (Figure 4.4.5a). After 2-4 weeks, cells in control wells that were not infected with ADV began to show faint localized random GFP expression as well. In contrast, untransfected A549 cells did not show any fluorescence, even cells that were 4 weeks old. The factor that seemed to correlate with the presence of this faint GFP expression was the overall health of the cells. Cells that were infected with ADV did show some fluorescence when uninfected cells did not, but uninfected cells that were allowed to grow without reseeding and proper feeding also showed GFP expression when they became unhealthy. To test this theory specifically, some of the clones were infected with ADV, and overlayed with the agarose media used in the PA to keep new ADV particles localized to their site of infection. The quantity of GFP expression was not significantly greater around the periphery of the plaques where greater ADV infection would be occurring. Areas where the cells were more crowded and stressed had greater GFP expression.

The entire process of creating stably transfected cells and testing them for ADV-dependent GFP expression was actually performed in two rounds. In the second round, efforts were made to increase transfection efficiency and to use cells that were recently

passaged that were actively dividing during the transfection to increase the likelihood of successful stable plasmid incorporation into the cell genome. The results were identical even for the second round. In order to determine if the stable cell clones being used contained the E2 promoter sequence, a few of them were tested using colony PCR. The result, illustrated in Figure 4.4.5b, shows that they did contain the promoter sequence. The PCR result proved that the cells also contained the E2 sequence addition to the neomycin resistance from the transfected plasmid. The project was halted at this point since it seems that alternate methods must be employed to achieve the desired cell line.

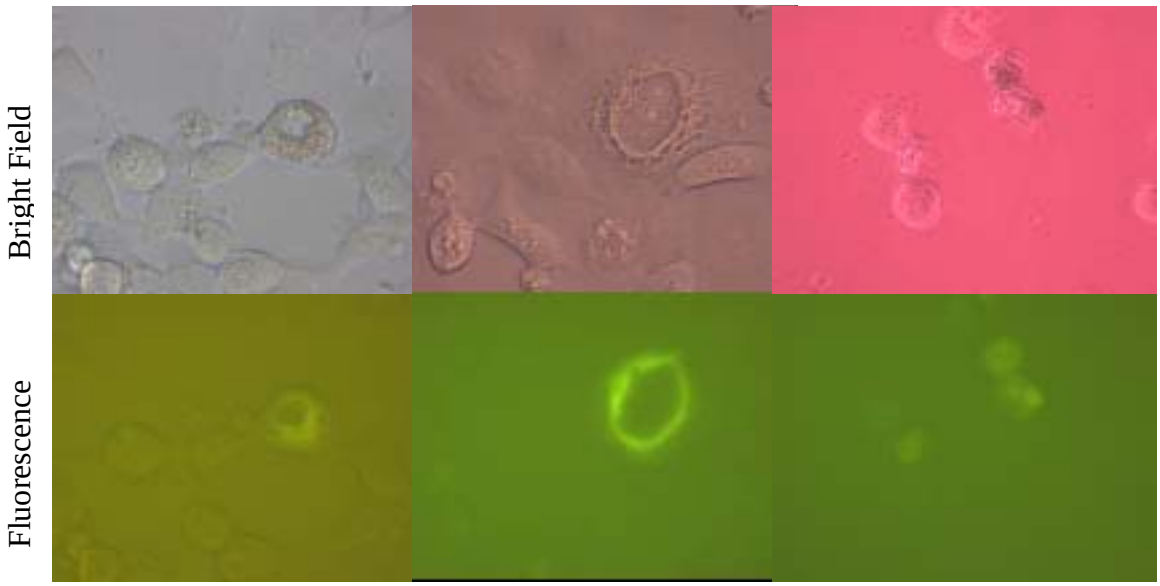
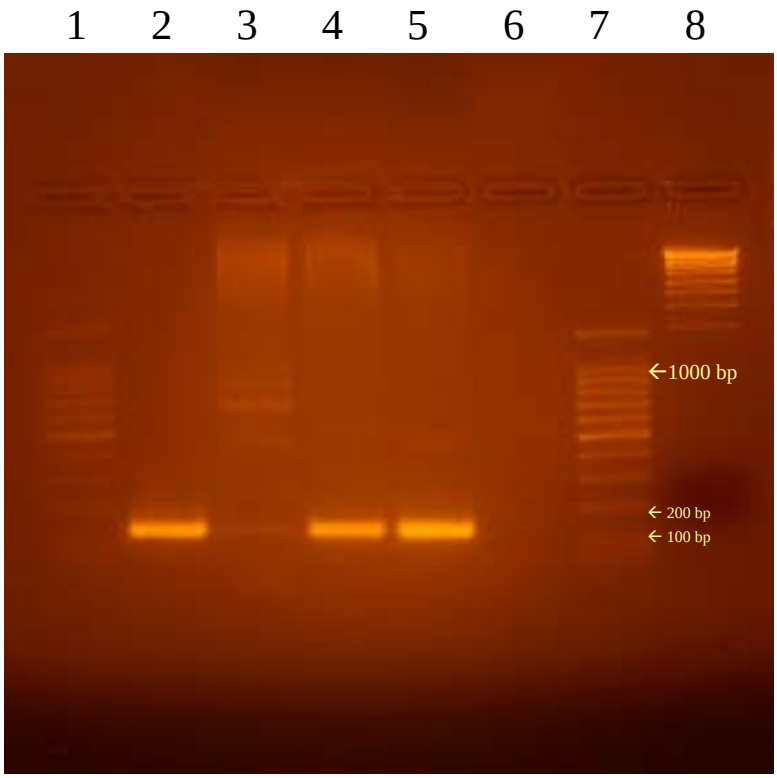


Figure 4.4.5a Images Illustrating Faint Localized GFP Expression in ADV Infected Stable Clones



1, 7, 8 MW markers
2 Positive Control, ADV infected A549 cells
3 Negative Control, A549 cells
4 Stably Transfected Clone W
5 Stably Transfected Clone N
6 Negative Control, No DNA

Figure 4.4.5b Colony PCR to Verify Promoter Presence in Stably Transfected Clones

Chapter 5

CONCLUSIONS AND FUTURE WORK

Section 5.1 Using Adenovirus that Constitutively Expresses GFP for Time-Kill Testing

In conclusion, performing anti-ADV TK testing by measuring fluorescence of recombinant ADV that constitutively expresses GFP (ADV-GFP) represents a significant assay improvement over traditional methods that evaluate CPE of *wt* ADV strains by CCID50 or PA. Both the traditional PA method and the new ADV-GFP method demonstrated similar LR results for solutions of ethanol and bleach. The most significant benefit of using the ADV-GFP instead of *wt* ADV was the reduction in time required to obtain the results with a decrease from at least 7 days to only 2 days. This was possible because the fluorescence detection of GFP expression occurs early in the replication of the virus and does not require the extensive virus production and induction of CPE that takes much longer.

In addition to the decrease in overall length of time needed to run the assay, use of ADV-GFP has several other advantages. Once the TK reaction mixture is neutralized and diluted, the traditional PA method requires several additional processing steps to enable plaque formation and to staining of the plaques before results can be obtained. In the ADV-GFP method the reaction mixture is added to the cells and no additional manipulations are needed. The total amount of labor required and the quantity of laboratory supplies and reagents that are needed is significantly less for the ADV-GFP method than is required for the PA method. Although the CCID50 method uses the same amount of supplies and reagents as the ADV-GFP method and does not need the

additional steps as the PA method, it still requires more time and is significantly less precise than the ADV-GFP method. The CCID50 method would need to be run 3-4 times to obtain an average that approaches a similar level of accuracy and precision as results that are obtained in only one run of either the PA or the ADV-GFP method. In addition, collecting results by rating CPE on CCID50 plates is more difficult because it is more subjective than merely counting green fluorescent cells from ADV-GFP plates. Thus the ADV-GFP method costs less to perform and is easier to do than either the PA or the CCID50 method.

Other advantages of using ADV-GFP are that it is more amenable to automation and that it yields relatively more objective results than either the PA or CCID50 methods. The ADV-GFP method also reduces requirement for a subjective interpretation and the skilled technician performing the test. When reading PA plates, the ability to accurately quantify the number of plaques can be adversely affected by the quality of staining, the density and adherence properties of the cells, and incubation conditions. The plaques often overlap one another and they can be of various sizes such that the person counting the plaques must use some subjective interpretation while collecting the results. In the CCID50 method extensive experience and familiarity with the cell line being used is necessary to accurately and consistently evaluate the extent of CPE in the cells. In contrast, however, utilization of fluorescence to collect data is considerably more objective and less dependent on a technician's skill and training level. Fluorescence detection instruments could be set up to automate the process of data collection thus completely removing the subjective interpretation of results.

Future research in this area should focus on testing the ADV-GFP method against other anti-ADV chemistries in addition to ethanol and bleach. It may also be desirable to create additional serotypes of ADV-GFP so that the efficacy of antiviral agents can be compared across various ADV types. This strategy does not need to be limited to merely ADV. Other viruses could be used in the same manner. It is also possible to use other fluorescent proteins which produce different colors for different viruses which would enable testing of products against cocktails of viruses at one time. This would be feasible for groups of viruses that were all able to infect the same cell type. These fluorescent protein expressing viruses could also be used in other anti-viral assays. For example, viral finger pad or hand washing studies as well as inanimate surface-based testing could be conducted with these viruses.

Use of automated fluorescence measurement is another area of future research for this application. A laboratory could be set up to do all anti-viral testing using fluorescent protein expressing viruses with automated fluorescence detection/quantitation systems. This innovation could significantly decrease the cost and turn around time for anti-viral product development.

Section 5.2 Development of Cells that Conditionally Express GFP upon Adenovirus Infection

An additional fluorescence-based assay utilizing cells created to fluoresce upon infection with *wt* ADV was conceived and tested to the point of proof of principle. A genetic construct was created with the promoter from an ADV early E2 gene upstream from a GFP gene. GFP expression was observed in HEK293 cells transiently transfected

with the plasmid. HEK293 cells express the E1a protein, a known activator of the E2 promoter. This demonstrated that GFP expression in the newly created construct was being controlled by the E2 promoter. More importantly, ADV infection was able to induce GFP expression in A549 cells which had been transiently transfected with the construct. This showed that the E2 promoter-GFP plasmid can be used as an indicator of adenovirus infection.

A549 cells were stably transfected with the plasmid construct and over 200 clones created. Presence of the E2 promoter region of several of the clones was verified using PCR amplification. All of the clones were infected with ADV and the expression of GFP monitored. So far none of the clones screened showed clear and consistent ADV infection dependent GFP expression. Some random faint GFP expression occurred in many of the stable clones, but it appears to be a nonspecific result that occurs most often in cells that are of poor health and it occured as often in cells that are unhealthy due to old age as it did in cells that are unhealthy due to ADV infection.

There are several possible explanations for the lack of bright fluorescence and GFP expression in the stable clones screened so far. Integration of only one or a small number of copies of the GFP expressing construct into the cell's genome may result in an insufficient production of molecules of GFP to generate sufficient fluorescence for detection. It is also possible that the constructs may be present in their entirety, but they may have integrated into locations that render them non-functional or unable to be expressed. Alternatively, only a portion of the construct may have integrated which either lacks the promoter or the complete GFP gene. The availability of E1a may be a factor as well. The quantity of E1a may be insufficient or the protein may be unavailable upon

ADV infection for activation of the E2-GFP construct. The E1a might not be expressed for a long enough time or in sufficient quantities to enable adequate activation of the GFP gene. The E1a is used by other promoters as well and therefore the newly inserted construct may be out-competed by stronger promoters or promoters that are in closer proximity to the E1a protein.

Future experiments should focus on completing the creation of a stable cell line that conditionally expresses GFP upon ADV infection. First, additional clones could be created and screened. This would increase the probability of selecting one in which the incorporation of the plasmid was amplified so that there are hundreds of copies of the plasmid and/or one in which the orientation and location of the insert is optimal. If that is not successful, several experiments may be conducted that would assist with understanding of the problem. The cell lines could be tested by delivering excess amounts of E1a protein into the cells to enhance the possibility of GFP expression from the ADV promoter. If the cells got brighter then we would know that part of the problem may be the availability of E1a during infection. The E1a could be obtained from HEK293 cells or by overexpression of the protein following co-transfection of the cells with a pE1a plasmid (used in 1985 Imperiale).

It may also be necessary to utilize alternate methods to create the stable cell line. Transduction could be used instead of transfection. It may be more efficient to utilize a viral construct to deliver the E2promoter-GFP construct directly into the cell's chromosome (perhaps a lentivirus or adenoassociated virus). We may also try using an epichromosome strategy which would enable semi-stable incorporation without integration into the chromosome. Based on the fact that we observed successful ADV

infection dependent GFP expression in cells transiently transfected with the construct, this strategy should work because it avoids the problems associated with chromosome integration. The major disadvantage, however, would be that it would be more difficult to get a cell line that is highly stable for extended lengths of time.

Once the stable cell line is created, it could have many other potential applications beyond just ADV TK testing. Any ADV work could be enhanced by the ability to quickly and easily titer the virus using the newly created cells. For example many gene therapy research projects utilize ADV. The cells could be used in laboratories involved in the identification of viruses as well. They could be used to identify ADV from respiratory samples for example. Other cells could be engineered using the same strategy. By employing different colors of fluorescent proteins governed by a different virus's early promoters (e.g. herpes simplex virus) a single cell line could be turned into a self-contained identification system allowing for the accurate identification of different infectious viruses from clinical or other samples. This would especially be useful to differentiate between viruses that are difficult to tell apart by current methods. TK and other viral test methods could also utilize this strategy and enable the testing of numerous viruses at one time.

Appendix I

DERIVATION OF THE LCM AND HCM MULTIPLICATION FACTORS

When quantifying infective virus by counting green cells expressing GFP it is necessary to examine the cells using fluorescence microscopy. Since it was impractical to count the total number of green cells in a well of a 12 well or 24 well tissue culture plate when viewing the cells under the microscope, it was necessary to develop a method to accurately estimate the total count in the wells based on a count obtained from a small portion of the well. Initial trials of possible counting methods revealed that wells with high concentrations of green cells needed to be counted by a different method than those with relatively low numbers of green cells. In addition, both 12 and 24 well plates were employed in the testing and therefore calculations needed to be done for both types of plates. Two counting methods were developed and applied to both 12 and 24 well formats. In addition, sometimes the cells or lighting in the laboratory was such that the cells were easier to count on 20X than on 10X magnification. The HCM was developed to be used when after counting one field of view under 10X or 20X magnification there were at least approximately 25 green cells visible. The total number of green cells in 5 fields of view (including one in the center of well, one above the center, one below the center, and one to the right and one to the left) were counted and averaged. If

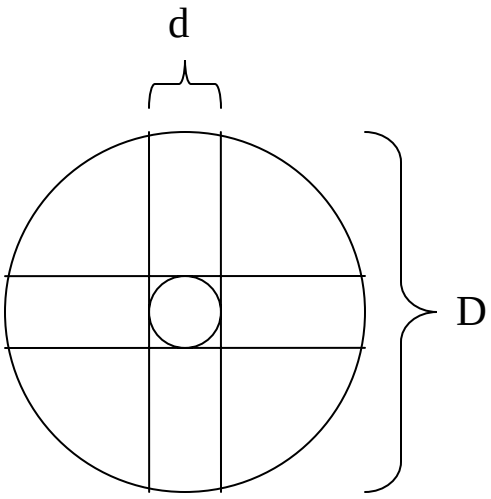


Figure A1 Plate Well and Field of View Diagram

after counting one field of view less than approximately 25 green cells were counted, the LCM was applied. The number of green cells in a vertical and a horizontal swipe across the field of view were totaled up for the low count method. Following is an explanation of how the HCM and LCM factors were determined. Figure A1 and Table A1 describe the starting variables including magnification, field of view and well diameters, and show how the HCM factors were calculated. The HCM factor is the ratio of the area of the actual well area to the area in one field of view under the microscope. Thus by multiplying the number of green cells counted in one field of view by the appropriate HCM based on the magnification and the well size one gets an approximation of the total number of green cells in the entire well. Usually about 5 fields of view were actually counted and averaged to get a more accurate estimate.

To calculate the LCM factors for the same magnifications and plate well sizes the area of the whole well (Aw) was divided by the total area swiped during the LCM counting (2 x Dd). The resulting LCM factors were 3.54 for a 24 well plate on 10X, 4.33 for a 12 well plate on 10X, 7.07 for a 24 well plate on 20X, and 8.64 for a 12 well plate on 20X. Spreadsheet templates were created such that the green cell counts could be entered and automatically converted to the estimated number of green cells in the whole well. Most often the results from a dilution using the HCM and the next lowest dilution using the LCM were averaged to achieve the final PFU.

Table A1 HCM Factor Determination

magnification	wells in plate	D	R	d	r	Aw	Af	HCM factor
		whole well diameter	whole well radius = D/2	field of view diameter	field of view radius = d/2	Area of the well = $Pi(R^2)$	Area of the field of view = $Pi(r^2)$	Aw/Af
10X	24	18	9	2	1	254.47	3.14	81
10X	12	22	11	2	1	380.13	3.14	121
20X	24	18	9	1	0.5	254.47	0.79	324
20X	12	22	11	1	0.5	380.13	0.79	484

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