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Genome-wide mapping of insertion sites essential for propagation of coxsackievirus A9

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Mutagenesis is crucial for understanding and harnessing genetic variation, providing a basis for evolutionary biology and experimental gene function studies. This thesis addresses the MuA transposase system, an effective species-non-specific transposition-based in vitro insertional mutagenesis strategy, intending to implement this system to generate a library of coxsackievirus A9 (CVA9) mutants. Like other members of the *Picornaviridae* family, CVA9 causes a variety of diseases such as hand-foot-and-mouth disease, respiratory maladies and even central nervous system infections. The lack of antiviral drugs and vaccines for this group of viruses necessitates further studies to advance our understanding of picornaviral pathogenesis and therapeutic targets.

The project's ultimate aim is to identify the genomic regions essential for the propagation of CVA9. This involves producing the required transposon mutagenesis system through the isolation, purification, and digestion of MuA plasmid DNA in order to assemble MuA-transposon complexes for mutagenesis. Furthermore, the thesis aims to check the functionality of the mutated pool of coxsackievirus A9 sequences and genome sequencing of viable virus mutants to map the mutated sites.

MuA and CVA9 plasmids were transformed into competent cells and purified using several maxiprep kits, initially, the manufacturer's protocol was followed. However, modifications were introduced to increase DNA concentration. Restriction enzyme mapping, PCR amplification, and transfection into mammalian cells were conducted. Successful infection and GFP expression in T7-BSR cells confirmed the functionality of cloned constructs.

This research establishes the fundamental components for using the Mu transposon system to create a library of mutated CVA9 (CVA9-eGFP vector) while highlighting the importance and impact of purification conditions, T7 RNA polymerase, primer design and transfection conditions in establishing a foundation for using the Mu transposition system in studying viral propagation. The findings lay the groundwork for future studies on picornavirus biology and the development of genetically modified viruses for pathogenicity studies and oncolytic virotherapy.

Keywords: Mutagenesis, picornavirus, MuA transposase, plasmid, bacteriophage Mu, oncolytic virotherapy, reverse genetics.

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1 Introduction

1.1 Background on mutagenesis

Mutagenesis refers to a process or event in which changes occur in an organism's genetic material (DNA or RNA). Mutagenesis could be a spontaneous event, the result of exposure to mutagens (physical or chemical mutation-inducing agents), or it can be performed experimentally in a laboratory environment. Moreover, the causes of mutagenesis can be endogenous or exogenous. Endogenous causes include molecular events such as errors in DNA replication, or repair mechanisms, Spontaneous base deamination or oxidative DNA damage. On the other hand, mutations caused by exposure to exogenous factors such as ionizing radiation (IR), ultraviolet (UV) radiation, alkylating agents and aromatic amines or other toxins are considered exogenous causes of mutagenesis (Durland & Ahmadian-Moghadam, 2024).

In the context of evolutionary biology, mutagenesis is the driving force behind biological evolution. This is because, mutations are the ultimate source of all genetic variation (Barton, 2010). Mutagenesis provides genetic diversity, which is the essential component of evolution by natural selection. Alterations in the genetic information of an organism may confer alterations in the phenotypic characteristics of the organism thus affecting its fitness and survival. Advantageous mutations, the ones resulting in a higher fitness, are more likely to be passed to offspring, thereby increasing their frequency in the population as they are favored by natural selection (Durland & Ahmadian-Moghadam, 2024; Lenski, 2017).

Mutagenesis, as a molecular biology technique, is the process of deliberately introducing changes into a DNA sequence. Such changes may be point mutations (mutations of a single nucleotide), or introductions or deletions of larger DNA fragments from a gene or even genome. As a laboratory technique, mutagenesis plays a very important role in our understanding of biology, as different mutagenesis strategies have been used to investigate the functions of genes (Clements et al., 2009), and proteins (Nisthal et al., 2019). Moreover, Mutagenesis has also been employed to understand the origin of genetic diseases and in disease modeling (Gondo et al., 2011). Additionally, Induced mutagenesis approaches are one of the most efficient tools that have been utilized in plant breeding (Chaudhary et al., 2019). More evidently, mutagenesis strategies are used in evolutionary studies as they induce genetic diversity, mimicking natural processes that drive population evolution. These techniques enable the study of the mechanisms

behind adaptation and speciation, thus revealing the genetic basis of evolutionary changes (MacLean et al., 2013; Park & Kim, 2021).

1.2 Overview of mutagenesis techniques in molecular biology

Recent progress in molecular biology resulted in a spectrum of mutagenesis techniques tailored to varying research needs. Although mutagenesis techniques were initially completely random, the development of site-directed mutagenesis allowed for more precision in manipulating specific sequences within the genome. Nevertheless, the aim of the work often defines the method to be used. In addition, and subject to mutagenesis technique an efficient high-throughput method is often required for the analysis of mutant repertoire.

1.2.1 Random mutagenesis techniques

While early mutagenesis techniques were predominantly random, inducing changes across the genome without a predetermined sequence specificity, they still remain powerful tools for generating mutant libraries with diverse properties distinct from that of the wild-type (Labrou, 2010). Several methodologies have been implemented to achieve random mutagenesis with the use of mutagens being perhaps the most classical method in which cells or organisms are exposed to mutagens, such as UV radiation or mutagenic chemicals, resulting in several mutations which are typically followed by selecting the desired mutants (Stottmann & Beier, 2014). Error-prone PCR is another commonly employed method for random mutagenesis. For instance, by the use of DNA polymerases with low fidelity such as Taq DNA polymerase (Wilson & Keefe, 2001). Additionally, modifying external conditions in which the PCR reaction is conducted such as buffer composition increases the misincorporation rate of nucleotides. Other frequently used methods for random mutagenesis include Saturation mutagenesis (Siloto & Weselake, 2012) and the propagation of cloned genes through mutator strains such as *Escherichia coli* XL1-red (Muteeb & Sen, 2010).

1.2.2 Site-directed mutagenesis techniques

Site-directed mutagenesis is used to modify plasmid DNA at specific locations. The advent of such techniques enabled researchers to cause predetermined specific amino acid changes and thus allowed one to study the relative impact that a certain amino acid has on protein conformation and function (Bachman, 2013). For site-directed mutagenesis to take place, a target gene or region of the genome and its nucleotide sequence must be known. Once the target

gene is specified, the basic procedure demands the synthesis of a short primer of 20 to 30 base pairs complementary to the target sequence but containing the desired mutation.

Several approaches have been used to achieve Site-directed mutagenesis including both PCR and non-PCR, as well as *in vivo* and *in vitro* techniques. Nevertheless, the general protocol involves several key steps. First, separating the two strands of the DNA template, using heat for example. The next step would be the annealing of the primer to a single strand of the template, allowing the synthesis of a complementary strand using DNA polymerase. Subsequent rounds of replication will produce DNA strands with the mutated site. At last, the desired mutants are selected using one of several possible screening methods such as DNA sequencing, to confirm the presence of the mutation (Durland & Ahmadian-Moghadam, 2024).

1.2.3 CRISPR-Cas9

The rise of CRISPR-Cas9 marks a monumental shift in biomedical research as this unique mutagenesis technology allows us to modify, in a relatively simple and rapid manner, the genome by removing, adding or altering sections of the DNA sequence. This genome-editing tool was derived from bacterial defenses against viruses and foreign plasmids. This prokaryotic immune system was co-opted in molecular biology as a powerful and versatile platform for genome engineering.

At its core, the system is dependent on two essential components: gRNA which is a guide RNA that binds to the target DNA sequence, and a Cas enzyme which is an endonuclease that causes double-stranded DNA breaks. These site-specific dsDNA breaks induce two of the cell's major endogenous repair mechanisms, which can be exploited for DNA modification. Nonhomologous end-joining (NHEJ) is a rapid but error-prone repair mechanism that can cause small insertions and deletions, resulting in frameshift mutations. This is mainly used in knock-out strategies. Alternatively, dsDNA breaks which are repaired by homology-directed repair (HDR), can be employed to achieve precise genome editing by inserting exogenous DNA sequences HDR approaches are more versatile than NHEJ since they can be used in both knock-in and knock-out strategies as well as tagging inserted sequences with marker genes (Durland & Ahmadian-Moghadam, 2024; Hryhorowicz et al., 2017).

1.2.4 Insertional mutagenesis techniques

Insertional mutagenesis refers to the process of integrating exogenous genetic material, one or more base pairs, into a genome altering its genetic sequence and causing an insertion mutation (Durland & Ahmadian-Moghadam, 2024). This process typically results in disruptions or changes in gene expression. Consequently, insertional mutagenesis is a powerful tool for studying gene function, identifying regulatory elements, and generating mutant libraries for genetic screens. Traditionally, insertional mutagenesis has been mediated through the use of viruses or transposons.

Viral-mediated Insertional Mutagenesis utilizes viruses' natural ability to integrate their genomes into the genomes of their host cells in order to replicate, subsequently, several viruses like Retroviruses, lentiviruses, and adenoviruses have been commonly used as vectors that would deliver and integrate the insert of interest into the targeted genome. Besides its usefulness in studying gene function, Viral-mediated Insertional Mutagenesis has been particularly useful for identifying cancer-associated genes (Uren et al., 2005).

Transposon-mediated Insertional Mutagenesis strategies harness the distinctive properties of transposable elements, DNA sequences capable of relocating within a genome. In this approach, transposons are utilized as elements inserted randomly into the genome. Such random integration of transposon DNA can induce alterations in gene expression and function, rendering it a valuable tool for genetic studies (Freed, 2017).

1.3 MuA

The Bacteriophage Mu employs a DNA transposition system for its replication and it is the first transposition system for which an in vitro transposition reaction was established, making it one of the most extensively studied mobile genetic elements (Mizuuchi, 1983).

The phage Mu encodes the MuA transposase protein, a member of the retroviral integrase superfamily (RISF) (Rasila et al., 2018). Serving as a pivotal catalyst., this protein is involved in both DNA cleavage and integration reactions, ultimately causing the integration of the transposon DNA into the target DNA.

In the natural context, the Mu transposition reaction would require the formation of a higher-order nucleoprotein complex called a transpososome which consists of four MuA molecules bound to the transposon at the two end sequences including MuA binding sites. The catalyzation

reaction would also involve another phage-encoded targeting protein called MuB, host-encoded DNA architectural proteins (HU and IHF), other DNA sequences such as additional MuA binding sites and a transpositional enhancer and the host-encoded ClpX protein, a factor responsible for restructuring the transpososome to facilitate its disassembly (Rasila et al., 2018).

In an in vitro setting, the assembly of efficient transpososomes could be carried out at a more minimalistic setup, requiring primarily the MuA transposase and suitable DNA substrates (Haapa, Taira, et al., 1999). Furthermore, it has been shown that the minimal in vitro Mu transposition system is highly efficient and displays very low target site selectivity. Additionally, previous studies demonstrated that the minimal setup is a more advantageous molecular biology tool for certain applications. For instance, a MuA transposase-based system is superior for a random transposon insertional mutagenesis, as the inclusion of the MuB transposition-targeting protein in the transposition reaction leads to regional target site preferences (Haapa, Taira, et al., 1999). As of now, Mu in vitro transposition-based strategies have been employed in various endeavors of molecular biology (Haapa, Suomalainen, et al., 1999; Laurent et al., 2000) and genetics/genomics studies(Lamberg et al., 2002; Vilen et al., 2003). Additionally, the adaptability of Mu in vitro transposition-based strategies as a flexible species-non-specific method renders it highly valuable, enabling its application across diverse organisms' genomes, including bacteria (Lamberg et al., 2002), yeast (Paatero et al., 2008), mammalian cells (Paatero et al., 2008), and even viruses (Kekarainen et al., 2002; Laurent et al., 2000).

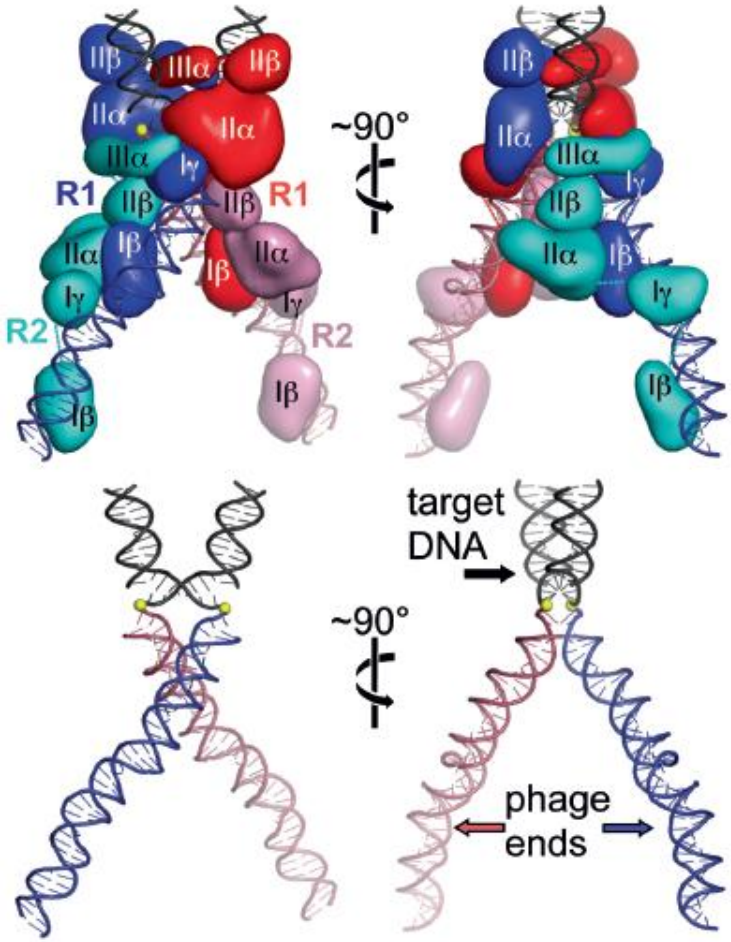

Figure 1. Illustrates the structure of the Mu transpososome from different perspectives. The upper part of the figure illustrates individual protein domains of the Mu transpososome as smoothed surfaces. The lower part of the figure presents the same structure with the protein domains removed, and the scissile phosphates are represented as yellow spheres.
Adapted from (Rasila et al., 2018).

To illustrate the functionality of the MuA system in protein functional analysis, Poussu et al. (2004) generated an extensive library of mutants within the alpha-complementing domain of *Escherichia coli* beta-galactosidase by using an insertional pentapeptide mutagenesis strategy based on MuA system in vitro DNA transposition. 15 base pairs were inserted randomly along the target sequence, resulting in several mutant proteins with each mutant having an additional five amino acids within its structure. Although most of the insertions were tolerated, some sites showed sensitivity to mutations, revealing regions of functional importance within the protein structure. Additionally, some mutant proteins displayed temperature sensitivity, implying the potential utility of the method for creating conditional phenotypes. Thus, the method should be applicable to any cloned protein-encoding gene (Poussu et al., 2004).

A more recent study by Kiljunen et al. (2014) exploited a MuA-transposase-catalyzed in vitro transposition reaction and combined it with *in vivo* gene targeting by homologous recombination, to produce a comprehensive transposon insertion mutant library for the model archaeon, *Haloferax volcanii*, and thus extending the application of Mu transposition mutagenesis into archaea. The methodology described in this study proved to be universal and applicable to other archaeal species, providing an efficient strategy for producing Insertion mutant libraries aimed at gene discovery in a research area where archaea are underrepresented.

Several studies have been conducted using viruses as targets. Viruses are good targets for mutagenesis since random mutagenesis together with appropriate screening methods allows the identification of functional sites in viral genomes and thus may reveal sites that are important for virus evolution, tropism and pathogenesis. Vilen et al. (2003), demonstrated the utility of the bacteriophage Mu-derived in vitro DNA transposition system for modification and functional characterization of bacterial virus genomes utilizing bacteriophage PRD1 as the model phage for this study (Vilen et al., 2003). Similarly, Krupovič et al. (2006) used the transposon insertional mutagenesis strategy for the genome characterization of lipid-containing marine bacteriophage PM2.

Besides bacteriophages, other viruses have been also studied using the Mu-derived in vitro DNA transposition system. For example, Laurent et al. (2000) focused on the human immunodeficiency virus type 1 (HIV-1) conducting a detailed analysis of the functional characteristics of the 1,000-nucleotide segment at the 5′ end of the virus genome. The study utilized 134 independent insertion mutations to assess their impact on RNA stability, viral particle assembly and release, and host cell entry. The results confirmed the previously known associated functional features of the target region of the HIV genome while also providing evidence for several novel features crucial for viral RNA stability, packaging, and replication (Laurent et al., 2000). In a similar setup, Kekarainen et al. (2002) used the insertional mutagenesis strategy to perform a complete functional mapping for the *Potato virus* A genome. With the generation of a comprehensive library of mutants using 15-bp insertions, the study revealed more than 300 sites essential for virus propagation, including several sites that had not been previously associated with any viral function. Furthermore, the study pointed out the suitability of several genomic regions for subsequent genetic manipulations, as these regions were more tolerant of insertional mutagenesis (Kekarainen et al., 2002).

These studies show that the Phage Mu transposition-based strategies have significantly contributed to genetics and molecular biology research, enabling researchers to generate comprehensive mutant libraries, identify essential genetic elements, and uncover functional characteristics in various biological contexts. Moreover, the discussed studies emphasize the wide-ranging applicability of this technique, spanning from bacteria to archaea and viruses, hence establishing it as a powerful transposon insertional mutagenesis strategy.

With this foundation in mind, we now turn our attention to picornaviruses, with a specific emphasis on coxsackievirus A9 (CVA9), to explore how the Mu transposition system can be applied in studying various aspects of this pathogen.

1.4 Coxsackievirus A9

Coxsackievirus A9 (CVA9) belongs to the family *Picornaviridae*, which is a large family of small nonenveloped viruses with an infectious single-stranded, positive sense RNA genome enclosed in an Icosahedral protein capsid. The *Picornaviridae* family is very diverse with 158 species assigned to 68 genera (https://www.picornaviridae.com). Among these, is the genus *Enterovirus* which includes some of the most common and important human pathogens such as rhinoviruses, polioviruses, echoviruses and coxsackieviruses. CVA9 belongs to so-called non-polio human enteroviruses (NPEV), which exemplifies the rest of enteroviruses in a post-polio era that requires attention. Like many NPEVs, CVA9 causes a variety of diseases such as hand-foot-and-mouth disease, respiratory maladies and even central nervous system infections (Tuthill et al., 2010).

1.5 Virus structure

Enterovirus particles are small in size (28 nm in diameter) and have no envelope. The viral RNA genome is enclosed in an Icosahedral protein capsid. Like most picornaviruses, the capsid is made of 60 copies of each of the capsid proteins VP1 to VP4, with the exterior part of the capsid being made of VP1-3, while the interior surface is composed of VP4. The icosahedral capsid of enteroviruses is composed of 12 pentameric units. The pentamer contains five triangular protomeric subunits, with each protomer formed by a VPs cluster (VPs 1-3), as shown in Figure 2.

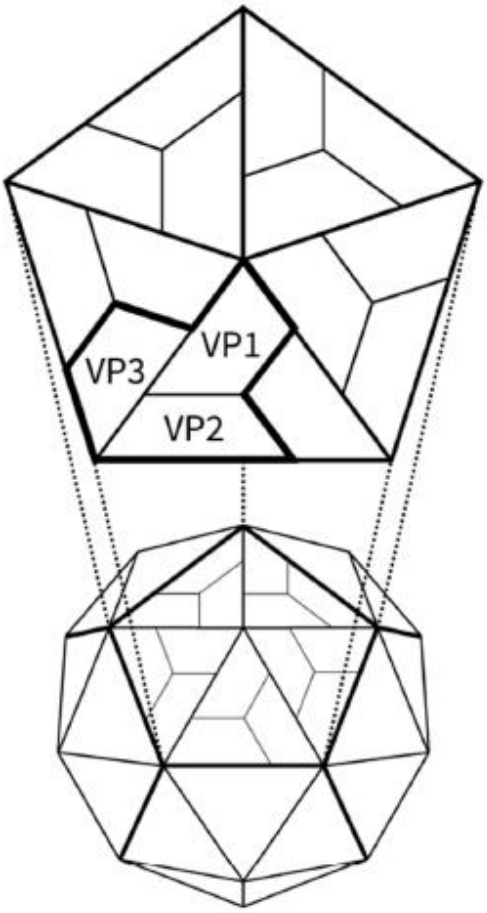

Figure 2. Picornavirus particle structure. The icosahedral capsid comprises 12 pentameric units, each consisting of five protomeric subunits arranged around five-fold symmetry axes. Surface-exposed capsid proteins VP1, VP2, and VP3 are indicated.
Adapted from (Ylä-Pelto et al., 2016).

Viral capsid proteins contain specific motifs that facilitate the binding of the virus to cell surface receptors, initiating the replication cycle. Notably, many enterovirus receptors are overexpressed in cancer cells, making native enteroviruses potential tools for oncolytic virotherapy. For example, coxsackievirus A9 (CV-A9) utilizes integrins as receptors for entering cells, by binding via the RGD motif located in the VP1 capsid protein. Thus, a CV-A9 vector could prove useful when targeting cancer cells that overexpress integrins such as the αVβ3 integrin (Tuthill et al., 2010; Ylä-Pelto et al., 2016).

1.6 Viral genome

The term picornavirus originates from 'pico', signifying small, and 'RNA', referring to the ribonucleic acid genome, thus 'picornavirus' literally translates to small RNA virus. True to

their name, these viruses harbor an RNA-based genome, precisely a 7-9 kb infectious single-stranded, positive-sense RNA genome. Like other RNA viruses, picornaviruses have a high mutation rate due to the lack of proofreading activity during genome replication. CVA9 specifically has a viral RNA of approximately 7.5 kb (Zhao et al., 2022). the RNA genome functions as an mRNA, the genetic information is translated into an extensive polyprotein via the internal ribosome entry site (IRES) translation mechanism. At the beginning of the genome, there is a connected peptide called VPg, which serves as an initiator for RNA synthesis and regulates the translation of the genome. a linked peptide known as VPg is positioned at the 5'-end of the genome, serving as a primer for RNA synthesis and as a regulating for translation of the genome. At the 3'- opposite end, a poly(A) tail ensures the stability of the genome structure. Additionally, both ends of the genome have untranslated regions (UTRs) which contain RNA secondary structures-associated elements. The presence of a single open reading frame in between these ends allows for whole genome translation into a single polyprotein. The capsid protein genes are located in the P1 region at the 5'-end of the genome, whereas the P2 and P3 regions at the 3' end house genes encoding for viral replication proteins. Subsequently, the polyprotein is auto-catalytically cleaved into functional structural and non-structural proteins by viral-encoded proteases, resulting in virus replication and ultimately the formation of the complete virus particles (Tuthill et al., 2010; Ylä-Pelto et al., 2016).

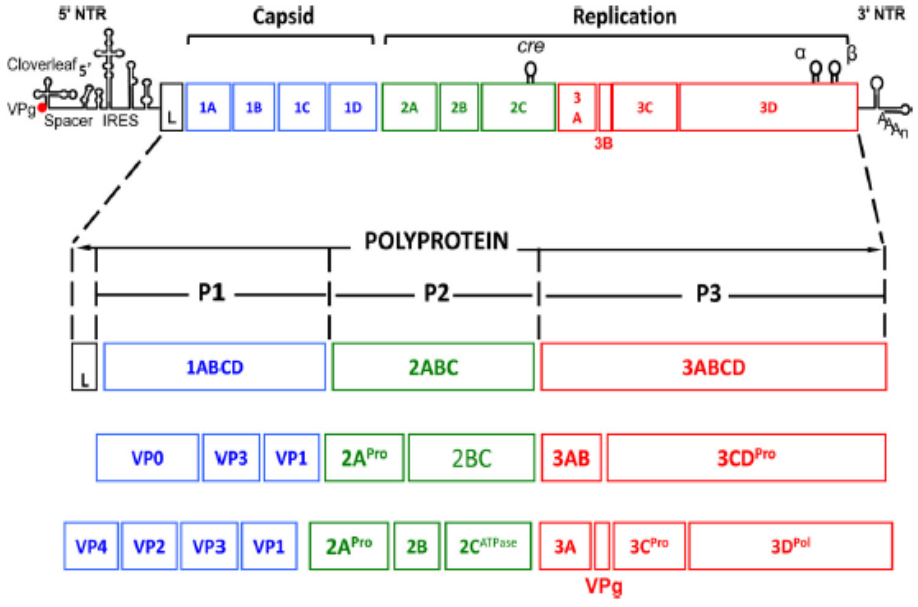

Figure 3. Genome structure of picornavirus and polyprotein processing. The picornavirus genome is a linear single-strand RNA ranging from 7.1 to 8.9 kb in length. The genome starts with a 5' linker peptide VPg and ends with a 3' poly(A) tail. Following this are the structural

and replication genes, arranged in a single open reading frame, which produces a single polyprotein. This polyprotein is then cleaved into individual protein products by viral proteases. Adapted from (Jiang et al., 2014).

1.7 Viral replication

Replication of picornaviruses takes place in the cell cytoplasm and their replication cycle could be divided into different stages (https://basicmedicalkey.com/picornaviridae-the-viruses-and-their-replication/). First, the virus gains entry to the cell by binding to a cell receptor, such as integrins and glycoproteins, thanks to viral capsid proteins. These proteins have specific motifs that facilitate binding to the receptors on the cell surface. After attachment, the virus is internalized into the host cell through endocytosis or other mechanisms. Once inside the cell, the viral capsid undergoes structural changes, allowing the RNA genome to be uncoated and thus released into the cytoplasm where it functions as an mRNA. Unlike most host-cell mRNAs, the picornavirus genomic RNA lacks a standard 5' cap structure which is utilized, in other viruses, to initiate the assembly of a ribosome onto the mRNA template (cap-dependent translation). As a consequence, the translation is dependent on the IRES region because of its ability to assemble ribosomes internally on the viral genome (Maclachlan & Dubovi, 2017). The resulting single polyprotein is then cleaved by viral proteinases into the individual structural and nonstructural proteins of the virus. Among the proteins synthesized are the viral RNA–dependent RNA polymerase and accessory proteins essential for genome replication and mRNA synthesis.

RNA synthesis occurs in viral protein-induced membranous vesicles, protecting the process from the host cell's defense mechanisms. Genome replication starts with the virus-encoded RNA-dependent RNA polymerase (RdRp) called $3D^{pol}$ making a negative-stranded intermediate RNA by copying the original positive strand. Afterward, more positive strands are made using the negative strand as a template. many of which are packaged into progeny virions. Ultimately, fully formed viral particles are discharged collectively from the cell as the cell structure breaks down. leading to lysis of the host cell (Maclachlan & Dubovi, 2017).

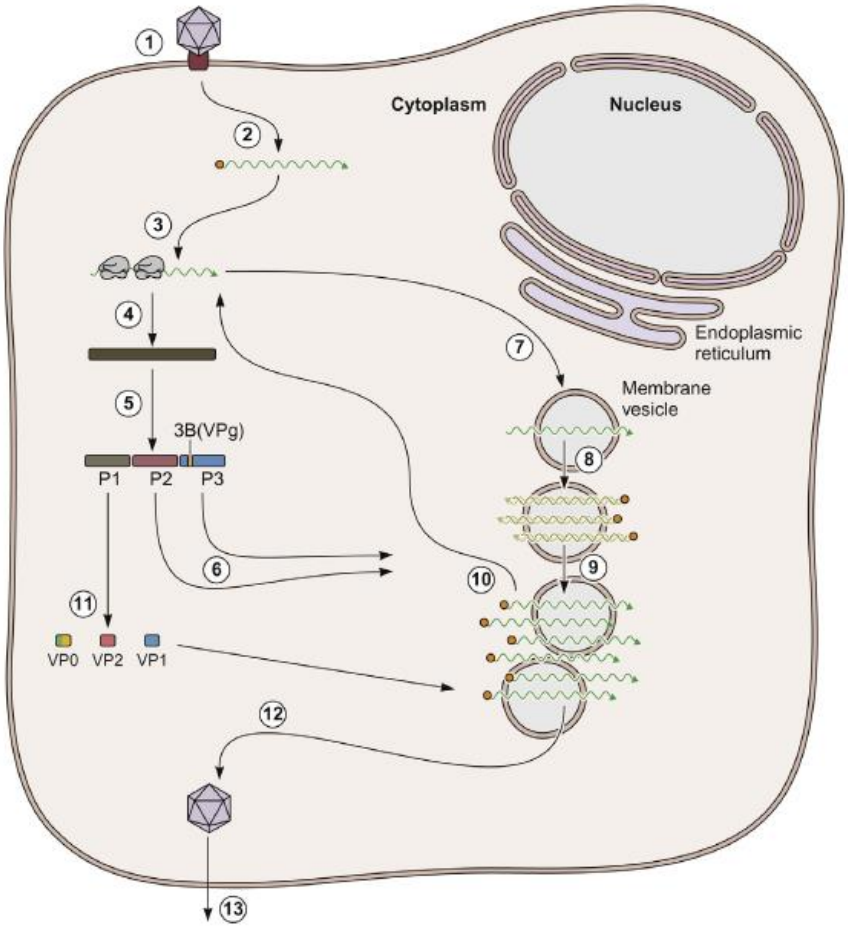

Figure 4. Picornavirus lifecycle. The Virus binds to a cellular receptor (1). Virus-receptor interactions induce the release of the viral genome in the cytoplasm (2). VPg linker protein is cleaved, allowing the cell ribosomes assembly on the genomic RNA (3). IRES-dependent translation produces a polyprotein (4). Viral proteases cleave the polyprotein into individual proteins (5). Proteins needed in the genome replication (6) and the genome template (7) are transported to membrane vesicles, where the positive sense genome serves as a template for the synthesis of complementary negative sense strands (8). These negative sense strands are then used as templates to generate the positive sense genomic RNA (9). Some of the newly produced RNAs are translated (10). In addition, certain viral proteins are used to form viral particles (11). Other genomic RNAs are packaged into these newly made viral particles (12). Complete virus particles are released causing cell lysis (13).
Adapted from (Maclachlan & Dubovi, 2017).

1.8 Virus rescue by using T7 RNA polymerase promoter tagging

In the context of this study, virus rescue is a reverse genetics technique, the study of gene function by assessing the phenotypic effects of specific genetic alterations. Virus rescue refers to the process of generating infectious viruses from cloned viral DNA or RNA sequences, allowing the study of viral gene function and replication and allowing for the development of

genetically modified viruses for various applications, including oncolytic virotherapy (Liu et al., 2021).

This research project tries to implement an *in vivo* T7-virus rescue system through the use of CVA9 genomic PCR amplicons tagged with a T7 promoter. Traditionally, Plasmid DNA or PCR amplicon-bearing viral genome is transcribed in vitro to make viral RNA, which is then transfected into cells. The same could in theory be accomplished by introducing the T7 promoter in the 5′-end of the viral genome during PCR. This is then recognized by T7 RNA polymerase produced by recombinant T7-BSR. T7RNA polymerase-driven transcription inside the cells leads to the production of infectious viral RNA and ultimately virus particles. The overall process aims to make the virus rescue process robust and easy-to-use.

1.9 Rationale and Motivation for this research

Like other viruses, many members of the *Picornaviridae* family pose significant threats to human and animal health, causing a variety of diseases such as hand-foot-and-mouth disease, respiratory maladies, and central nervous system infections. Moreover, the complexity and diversity within this family of viruses with 158 known species plus many unassigned viruses, calls for more in-depth research on various aspects of picornavirus biology.

As discussed earlier, several studies have used the Mu transposon-based insertional mutagenesis strategies to produce mutant libraries, pinpoint essential genetic elements, and uncover functional characteristics in diverse biological contexts in a variety of organisms and viruses. Thus, the use of such techniques to study specific aspects of an enterovirus is a tempting and promising endeavor.

Understanding the genetic makeup of CVA9 will not only advance our knowledge of picornavirus biology, but such research can pave the way for the development of genetically modified viruses for pathogenicity studies and even for the emerging field of oncolytic virotherapy. For instance, many of the protein receptors and/or attachment factors which enteroviruses use for cell binding and initiation of the replication cycle, are overexpressed in cancer cells. Knowing that receptor binding and the ability to replicate in specific target cells define the tropism and that cellular infection often results in a cytolytic response, i.e., disruption of the cells, makes enteroviruses such as coxsackievirus A9 prime candidates for oncolytic virotherapy (Ylä-Pelto et al., 2016).

1.10 Aims of the study

In the big scheme, the primary aim of this research is to utilize the Mu transposon system, a transposition-based in vitro insertional mutagenesis strategy to perform a genome-wide mapping of insertion sites essential for the propagation of coxsackievirus A9. Following mutagenesis, the viral cDNA clones are converted to PCR amplicons using T7-promoter-tagged primers, which allow virus rescue in compatible cells, and thus identification of the effects of random insertions. Genome sequencing is used to map the precise location of the mutations, which allows us to pinpoint their impacts on virus propagation and functionality. A specific aim is to identify genomic region, within the viral capsid, where one could insert extra genetic material without disturbing virus replication. For all of this to take place, one needs to:

- First, produce the needed transposon mutagenesis system, starting from MuA plasmid DNA isolation, purification, and digestion to the assembly of MuA-transposon complexes (in collaboration with Prof. Harri Savilahti, Dept. Biology, Univ. Turku).
- Second, check the functionality of the mutated pool of coxsackievirus A9 sequences by use of the T7-virus rescue system.
- Lastly, genome sequencing of viable functional virus mutants to identify the mutated sites.

2 Materials and Methods

2.1 Plasmids

MuA plasmid (pSTH11) for mutagenesis was obtained from Professor Harri Salvilahti (Department of Biology, University of Turku, Finland). The plasmid pSTH11, which carries a full copy of the MuA gene, has been described. (Haapa, Suomalainen, et al., 1999).

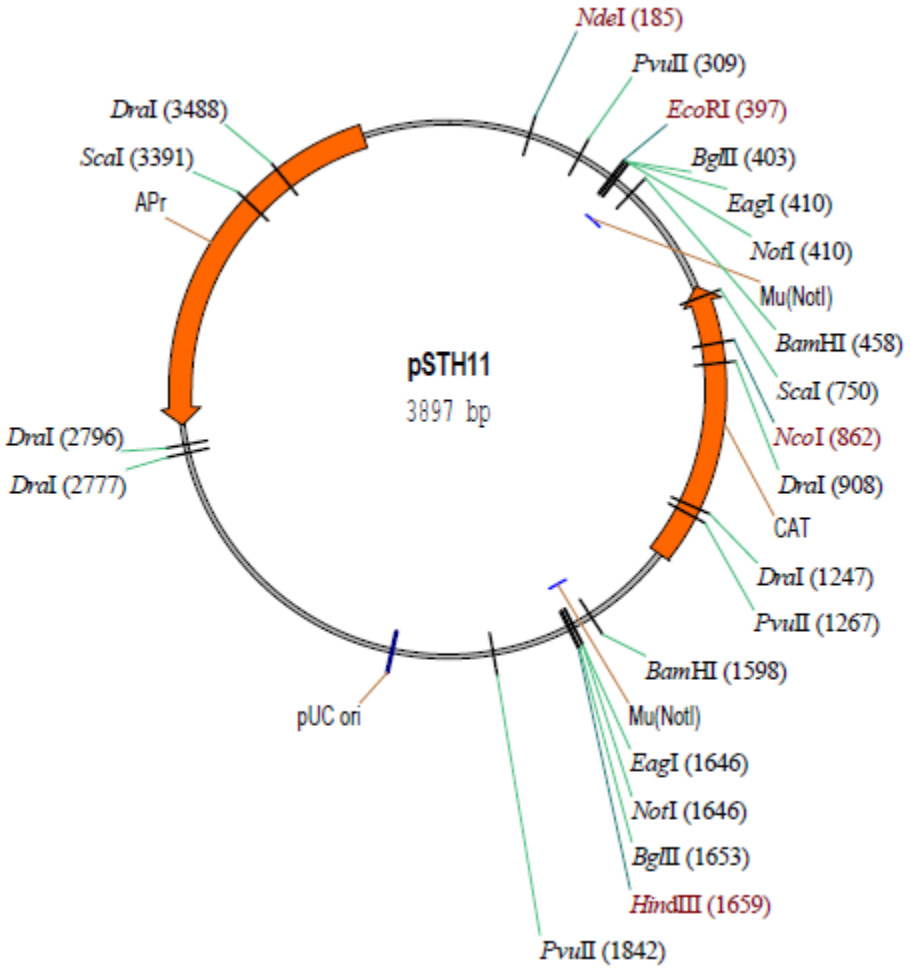

Figure 5. The pSTH11 plasmid contains a full copy of the MuA gene.

Plasmids containing the full genome of coxsackievirus A9 alone or with the green fluorescent protein gene (Heikkilä et al., 2011) were from the Picornavirus laboratory collection. Moreover, the study also employed two plasmids containing the T7 polymerase enzyme encoding gene. pCMV-T7Pol, a plasmid carrying a cytomegalovirus, or CMV promoter which was from Dr. Ralph Meyer (University of Tübingen, Germany), and pCMV/T7-T7pol, constructed by inserting HINDIII/BamHI fragment from Par3126 into corresponding sites of the pcDNA3

plasmid vector, was from Dr. Karl-Klaus Conzelmann (Federal Research Center for Virus Diseases of Animals, Tübingen, Germany). The details of each plasmid, including their properties and relevance to the study, are described in Table 1.

Table 1. Plasmid characteristics and purposes for the project.

Plasmid	Description/ Purpose for the project	Selection Marker	size	Reference/ Source
MuA plasmid (pSTH11)	Carries the MuA gene needed for the production of the transposon mutagenesis system.	Ampicillin resistance	3897 bp	(Haapa, Taira, et al., 1999)
pCVA9	Coxsackievirus A9 genome containing plasmid needed for the production of viral particles.	Ampicillin resistance	7500bp	Griggs strain, GenBank acc. no D00627
pCVA9-eGFP (6, 7, 11)	Plasmid containing CVA9 genome with the green fluorescent protein-encoding gene (eGFP) inserted into the VP1-2A junction of the CVA9 cDNA backbone plasmid.	Ampicillin resistance	8200bp	(Heikkilä et al., 2011)
pCMV-T7Pol	Plasmid containing the T7 polymerase enzyme encoding gene. Driven by a cytomegalovirus, or CMV promoter.	Kanamycin resistance	10297bp	(Meyer et al., 2004)
pCMV/T7-T7pol	A plasmid consisting of the T7 RNA polymerase gene driven by a CMV and T7 promoter.	Ampicillin resistance	8400bp	(Brisson et al., 1999)

2.2 Plasmid transformation

For plasmid transformation, 1µl of plasmid DNA was mixed with 25 µl of competent *E. coli* cells, OneShotTOP10, Invitrogen™ (www.thermofisher.com/order/catalog/product/C404010) and incubated for 30 min on ice. The cell-DNA mixture was subjected to a heat shock exactly for 45 seconds in a water bath at 42°C, followed by Incubation on ice for 2 min. Following the heat shock treatment, 250µl of SOC medium to the tubes and incubated at 37 °C for 30 min on

a shaker after which the cells were spread onto LB-agar plates supplemented with antibiotics, ampicillin or kanamycin depending on the viral plasmid (Table 1), the stock concentrations for ampicillin and kanamycin were 50 mg/mL, with final concentrations of 100 µg/mL for ampicillin and 25-50 µg/mL for kanamycin. Plates were incubated overnight at 37 ºC.

2.3 Cultivation of bacterial culture

To cultivate bacteria, single colonies were picked from each plate, using a pipette tip and transferred into 5 mL snap-cap Falcon tubes with each containing 5 mL Luria Broth (LB) supplemented with the appropriate antibiotic (Table 1) These tubes were then placed in a 37°C shaking incubator overnight, the shaker facilitates nutrient distribution throughout the culture medium, ensuring uniform growth.

Following overnight incubation, 1 ml of the bacterial culture from each snap-cap Falcon tube was transferred to separate Eppendorf tubes and centrifuged at 6000 rpm for 5 minutes to obtain a pellet. The supernatant was carefully removed, and the cell pellet was resuspended in a solution consisting of 40% glycerol- LB medium. Cell stocks were stored at -80 ºC.

The remaining 4 ml of bacterial culture was used for plasmid purification; cells were concentrated by centrifugation and the supernatant was removed. The obtained pellets were utilized for minipreps plasmid purification following the manufacturer's protocol (NucleoSpin Plasmid DNA Purification Kit, Macherey-Nagel). Plasmids were then digested to pre-screen for the correct plasmid size.

2.4 Plasmid Isolation

To purify large amounts of plasmid DNA from transformed bacteria, different commercial kits were used.

2.4.1 QIAGEN® Plasmid Plus Maxi Kit

200 ml of Luria-Bertani (LB) broth supplemented with the appropriate antibiotic (Table 1) was prepared in an Erlenmeyer flask. 100 µL of plasmid-containing cells, from the cell stock prepared earlier, was added to the flask. Subsequently, the flask content was allowed to incubate overnight in a shaker incubator at 250 rpm, +37ºC.

The cell suspension was divided into two centrifuge bottles and centrifuged at 10,000 rpm using a high-speed centrifuge, Beckman Coulter Avanti JA-14 rotor, for 10 minutes at +4°C. the supernatant was carefully removed to obtain a cell pellet which was used for the DNA purification following the manufacturer's protocol (QIAGEN® Plasmid Plus Maxi Kit).

2.4.2 Sigma GenElute™ HP Endotoxin-Free Plasmid Maxiprep Kit

In addition to the QIAGEN® Plasmid Plus Maxi Kit, large-volume purification of high-quality plasmid DNA was conducted using Sigma's GenElute™ HP Endotoxin-Free Plasmid Maxiprep Kit. For this, 50 µl of plasmid-containing cells, sourced from the previously prepared cell stock, was added to a 2 ml LB, supplemented with the appropriate antibiotic (Table 1), to make a 4-hour pre-culture in a shaker incubator at +37 ºC and 225 rpm. After that, the 2 ml pre-culture was transferred to a larger 200 ml LB medium and cultivated overnight (+37 ºC, 225 rpm). Plasmid DNA purification was carried out according to the manufacturer's protocol (Sigma GenElute™ HP Endotoxin-Free Plasmid Maxiprep Kit).

2.4.3 MACHEREY-NAGEL NucleoBond Xtra Maxi Plus kit for transfection-grade plasmid DNA

A third kit was used, MACHEREY-NAGEL NucleoBond Xtra Maxi Plus kit for transfection-grade plasmid DNA, for this time, the amount of the growth medium was upscaled using two Erlenmeyer bottles of 500 ml LB with each one being supplemented with the appropriate antibiotic (Table 1) and 75 plasmid-containing cells, sourced from the previously prepared cell stock. These two flasks were separated into four bottles and centrifuged using the same setup used for the previous kits. The resulting pellets were utilized in DNA purification following the manufacturer's protocol (MACHEREY-NAGEL NucleoBond Xtra Maxi Plus kit for transfection-grade plasmid DNA).

Four tubes of 15 ml of plasmid DNA eluted in an elution Buffer were obtained. The purification process was followed by an Isopropanol precipitation, commonly used for concentrating, desalting, and recovering nucleic acids in which 10 ml of room temperature Isopropanol was added to each tube and centrifuged at 10,000 rpm for 30 min at 4°C. The supernatant was carefully removed without disturbing the pellet which was washed by adding 10 ml of room temperature 70% ethanol to remove co-precipitated salt and replace isopropanol with the more volatile ethanol, making the DNA easier to redissolve. This was again followed by centrifugation, conserving the obtained pellet, air-drying the pellet for 20 min and redsolving

the DNA by adding 500 µl of a suitable buffer to each tube, I have used the Macherey-Nagel NucleoSpin Plasmid DNA Purification Kit, resulting into four tubes of 500 µl.

The concentration of purified plasmids was measured using the DeNovix DS-11 nanodrop spectrophotometer (DeNovix Inc., Wilmington, USA).

2.5 Restriction enzyme mapping of plasmids

Plasmid sequences were analyzed using the NEBCutter program from New England Biolabs (https://nc3.neb.com/NEBcutter/) in order to choose the appropriate restriction enzymes and to create a restriction map, essential for verifying the band sizes after DNA electrophoresis on an agarose gel.

For the MuA plasmid, Minipreps purification using NucleoSpin Plasmid DNA Purification Kit, Macherey-Nagel, were digested individually using NotI.

Similarly, the products of the Maxiprep purifications were also checked. However, additional restriction enzymes were utilized. The MuA plasmid DNA was digested individually using the following enzymes: NotI, BamHI, BglII, and EcoRI, HindIII, as well as an additional digestion with EcoRI + HindIII.

For the remaining plasmids, purification products were checked using the restriction enzyme ClaI for pCVA9, while pCVA9-eGFP was digested using NotI. Furthermore, pCMV-T7Pol (10297bp) (Meyer et al., 2004) was digested with Hind III and EcoRI, while pCMV/T7-T7pol (8400 bp) (Brisson et al., 1999) was checked using Hind III and EcoRI, as well as Hind III + BamHI.

A digestion master mix was prepared for each sample with the appropriate restriction enzyme (FastDigest enzymes from ThermoFisher Scientific) based on the following chart (n = number of samples).

Table 2. Digestion master mix preparation.

Reagent	**Volume (µl)**	**Master mix (example for 7 samples (n + 1 = 8))**
Plasmid (in a tube)	2	N/A
ddH20 (Milli-Q)	15.5	124 (15.5 x 8)
10 x buffer	2	16 (2 x 8)
R-enzyme	0.5	4 (0.5 x 8)
Total	**20**	144 (18 x 8. Note that 2 µl of plasmid is already added to the tube, and then 18 µl of master mix is added to make a total volume of 20 µl.)

After mixing the sample with the master mix, the mixture was incubated at 37°C for 30 minutes and then returned to ice. Thermal inactivation of the enzyme was performed, and the samples were again placed on ice. Table 3, summarizes the thermal inactivation protocol used for each enzyme.

Table 3. FastDigest enzymes and their thermal inactivation conditions.

FastDigest enzyme	NotI	BamHI	BglII	EcoRI	HindIII	ClaI
Thermal inactivation	80°C, 5 min	80°C, 5 min	N/A	80°C, 5 min	80°C, 10 min	N/A

2.6 Agarose gel electrophoresis

Agarose gel electrophoresis was conducted to check the DNA purification products following restriction enzyme digestion as well as to assess the success of the PCR reaction, using Tris-acetate-EDTA (TAE) buffer and 1% agarose gel. The PCR product samples were mixed with loading buffer (6X TriTrack DNA Loading Dye, Thermo Fisher Scientific). Midori Green Advance DNA Stain dye was added to the gel to make the DNA visible. In addition, GeneRuler DNA Ladder Mix (Thermo Fisher Scientific) and GeneRuler 100 bp Plus DNA Ladder (Thermo Fisher Scientific) served as a reference to determine band sizes. Electrophoresis was conducted at 100V until bands appropriately separated. The gels were imaged using Bio Rad Gel Doc XR+

Gel Documentation System (Bio-Rad Laboratories, Inc.) and analyzed using Image Lab (Bio-Rad Laboratories, Inc.) software.

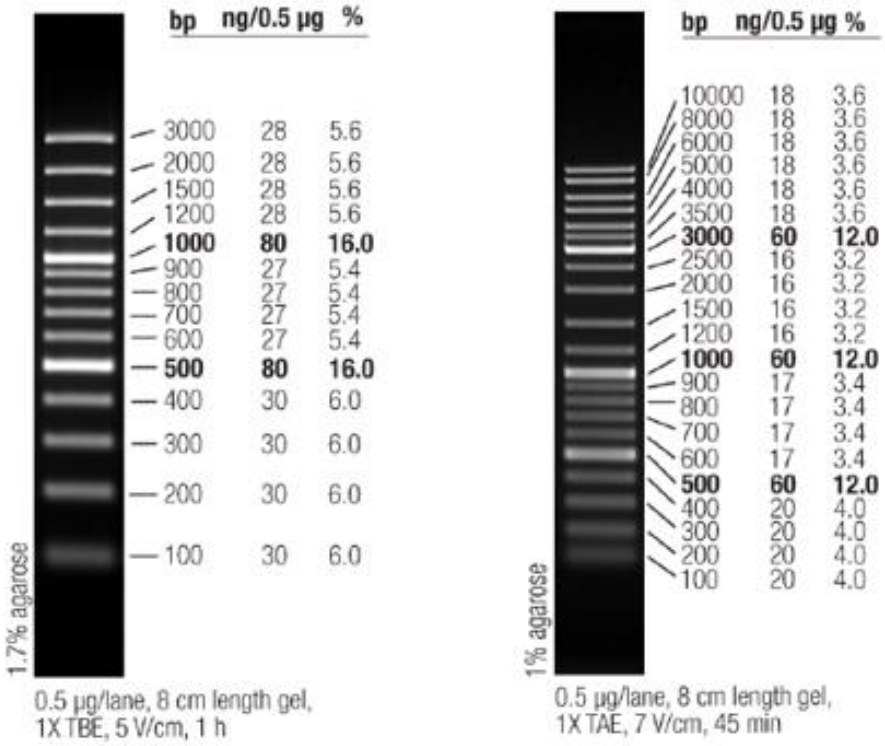

Figure 6. Gel electrophoresis markers. The figure displays the GeneRuler 100 bp Plus DNA Ladder on the left and the GeneRuler DNA Ladder Mix molecular weight marker on the right. These markers were used to assess the sizes of the bands formed in gel electrophoresis.

2.7 PCR

To amplify the CVA9 genome using pCVA9 a 5'-end primer, pCAV9gen1-F, was used. The primer pCVA9gen1-F contains a T7 RNA polymerase promoter (T7p) (Hughes et al., 1995), a complementary region for CVA9 following a three G residues construct. On the other hand, the 3'-end primer, pCAV9gen1-R, has a long T-tail in order to generate stable poly(A) tails. Three separate sets of reverse primers, along with one common forward primer, were used to amplify PCR amplicons, as shown in the table below.

Table 4. Primers used for PCR. Complementary section underline. F= forward R= reverse.

Primer	**Sequence**
pCAV9gen1-F	5'-TAATACGACTCACTATA GGGTTTAAAACAGCCTGTGGGTTGTTCCC-3'
pCAV9gen1-R	5'-TTTTTTTTTTTTTTTTTTT CCTCCGCACCGAATGCGG-3'
CVA9gen1-R_T7- T^{orig}	5′-AACCCCTTGGGGCCTCTAAACGGGTCTTGA GGGGTTTTTTGCU-3′
CVA9gen1-R_T7- T^{synt}	5′-AACCCCGCGGGGCCTCTTCGGGGGTCTCGC GGGGTTTTTTGCT-3′

Platinum SuperFi II DNA polymerase (Thermo Fisher Scientific, CAT# 12361010), a high-fidelity, long-range DNA polymerase enzyme, was used for long-PCR reactions. The reaction mix for the PCR enzyme is presented in Table 5.

Table 5. Reaction mix for PCR enzyme used.

Reagent	**Volume (µl)**
Nuclease-free water (ddH_2O)	12.2
5 x PCR buffer	4
dNTP mix (10 mM)	0.4 (200 µM)
F- primer (10 µM)	1 (0.5 µM)
R-primer (10 µM)	1 (0.5 µM)
Template DNA (10 ng)	1
Platinum SuperFi	0.4
Total volume	**20µl**

PCR reactions were carried out in a Veriti™ 96-Well Fast Thermal Cycler (Applied Biosystems™ by Thermo Fisher Scientific) following the cycling programs provided in the manufacturer's user manual, as outlined below.

Table 6. PCR Cycling Conditions.

	Temperature	Time	Cycles
Initial denaturation	98 °C	60 s	1
denaturation	98°C	15 s	30-40
annealing	67°C	20 s	30-40
extension	72°C	4 min	30-40
Final extension	72°C	7 min 30 s	1
hold	4°C		

The PCR reaction was checked with agarose gel electrophoresis using 2 µl of PCR amplicon. NucleoSpin® Gel and PCR Clean-up (MACHEREY-NAGEL, Düren, Germany) kit was used to purify the remaining amount of PCR products.

2.8 Cell transfection

To check the functionality and infectivity of the target, coxsackievirus A9. Both pCV-A9 and pCV-A9-eGFP (s), as well as the T7-PCR amplicons, were transfected into mammalian cells using two cell lines: BHK-21 (baby hamster kidney cells) and T7-BSR, BHK-21 derivative (Buchholz et al., 1999). These adherent cell lines were cultured in T-75 flasks at +37°C and 5% CO_2 in Dulbecco's minimal essential medium (DMEM medium, BioWhittaker, 12-709F) supplemented with 10% fetal bovine serum (FBS, GIBCO Life Technologies, USA).

The cells were periodically diluted and passaged every three days. Prior to transfection, cell viability and count were assessed using trypan blue exclusion via a TC20™ Automated Cell Counter (BIO-RAD). Transfection reactions were consistently carried out using Lipofectamine™ 3000 Reagent (Thermo Fisher Scientific, L3000075).

CV-A9 and CV-A9-eGFP (s) plasmids transfection reactions were carried out in 96-well (Perkin Elmer plates, 6005050) plates. For the plate containing T7-BSR cells, 20000 cells were added to each well of the 96 wells. The cells were incubated overnight at +37°C with 5% CO_2 until reaching 70-90% confluence. The growth medium for T7-BSR cells consisted of DMEM supplemented with 10% fetal bovine serum (FBS), 10 µg/ml gentamicin antibiotic (Biological Industries, 03-035-1B), and 200 µg/ml Gibco Geneticin antibiotic (Thermo Fisher, 10131035).

Similarly, for the plate containing BHK-21 cells, 20,000 cells/well were cultured overnight under similar conditions. The plasmids and T7-PCR amplicons were then transfected into the cells, with two parallel wells per plate. The transfection mixture was prepared according to the Lipofectamine™ 3000 Reagent manufacturer's instructions, with a final sample volume of 2.5 µl per well (in 1:1 ratio), containing 0.15 µl/well (1:1) Lipofectamine™ 3000 Reagent. Before adding the transfection mixture, the cell culture medium was replaced with 100 µl of serum-free DMEM medium. The final volume in each well was 110 µl.

A plasmid encoding the green fluorescent protein gene, pGFP (a gift from Dr. Jukka Alinikula, University of Turku) was used as a transfection control.

2.9 Imaging of transfected cells

After transfection, virus-infected cells were stained using the immunofluorescence staining (IFA) method. First, cells were fixed by adding 100 µl of 4% (para)formaldehyde (PFA) to each well and incubating at room temperature for 15 minutes. Then, the wells were washed twice with 100 µl PBS solution. Next, the cells were permeabilized by adding 100 µl of 0.2% Triton X-100 solution to each well and allowing it to incubate for 5-15 minutes at room temperature. After three washes with PBS, the cells were stained with a DAPI (4′,6-diamidino-2-phenylindole, Thermo Fisher Scientific) marker. The DAPI solution was diluted 1:2000 in a pre-made 3% BSA (bovine serum albumin)-PBS (phosphate-buffered saline) solution and added to the wells (60 µl per well). After shaking at room temperature in the dark for 10 minutes, the wells were washed twice more with PBS.

After cell nuclei staining, cells were imaged with an EVOS FL AUTO fluorescence microscope (Invitrogen, Thermo Fisher, AMAFD1000). Wells containing cells were imaged in a way, cell nuclei were imaged in the blue (DAPI mode) light channel, and cells transfected with CV-A9-eGFP were imaged in the green channel.

3 Results

3.1 Verification of plasmids

Minipreps plasmid purification products, following the manufacturer's protocol (NucleoSpin Plasmid DNA Purification Kit, Macherey-Nagel), were digested to pre-screen for the correct plasmid size.

For the MuA plasmid, the digestion of three samples, representing three different colonies, with the restriction enzyme NotI resulted, as shown in figure 7, in two fragments of 1200 bp, containing the MuA gene, and 2700 bp, containing the remaining plasmid backbone.

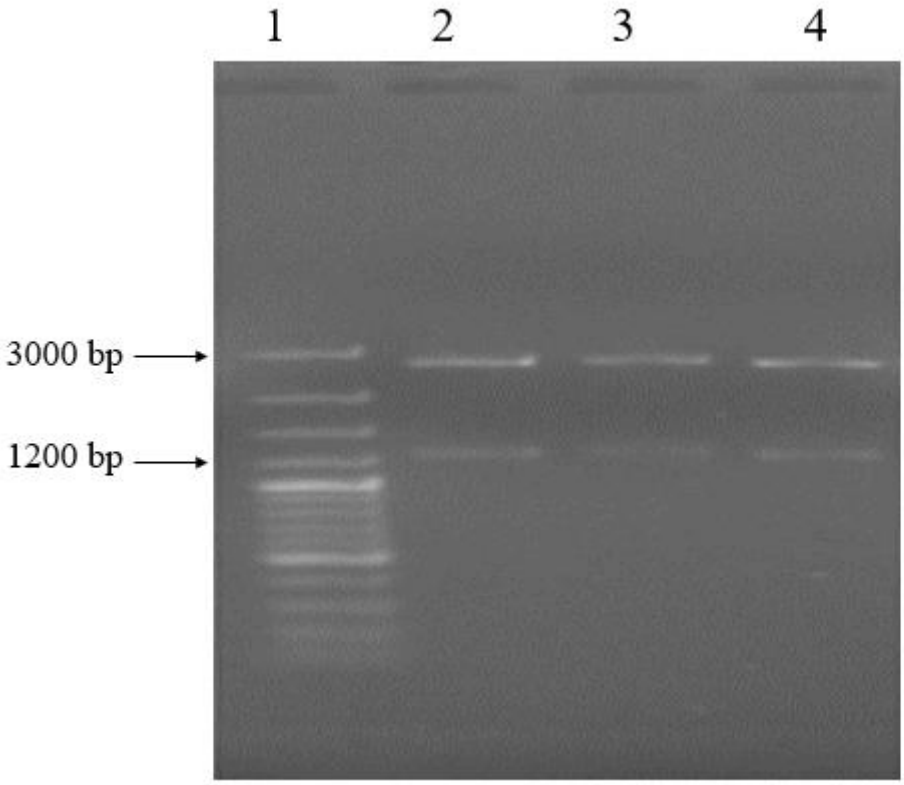

Figure 7. Restriction digestion analysis of MuA plasmid using NotI enzyme. Lane 1 contains the molecular weight ladder, GeneRuler 100 bp Plus DNA Ladder. Lanes 2 to 4 contain MuA plasmid samples. MuA gene (1200 bp) and plasmid backbone (2700 bp) are shown by arrows.

Consistently, samples 1 to 3, shown in lanes 2 to 4, displayed the expected banding pattern. with two distinct bands: the MuA gene at approximately 1200 bp and the plasmid backbone at approximately 2700 pb. Based on these results, the viral and cell stocks corresponding to samples 1 to 3 were selected for further large-scale maxiprep purification and subsequent experimentation.

The digestion of pCVA9 and pCVA9-eGFP (s), with ClaI and NotI FastDigest enzymes, respectively, resulted in a single band of 7500 bp for the pCVA9 (Fig. 8). This outcome is expected since ClaI is a single cutter for pCVA9, linearizing the plasmid, while the digestion of pCVA9-eGFP (s) with NotI produced two bands: one at 700 bp, corresponding to the eGFP gene, and another at 7500 bp, corresponding to the CVA9 genome.

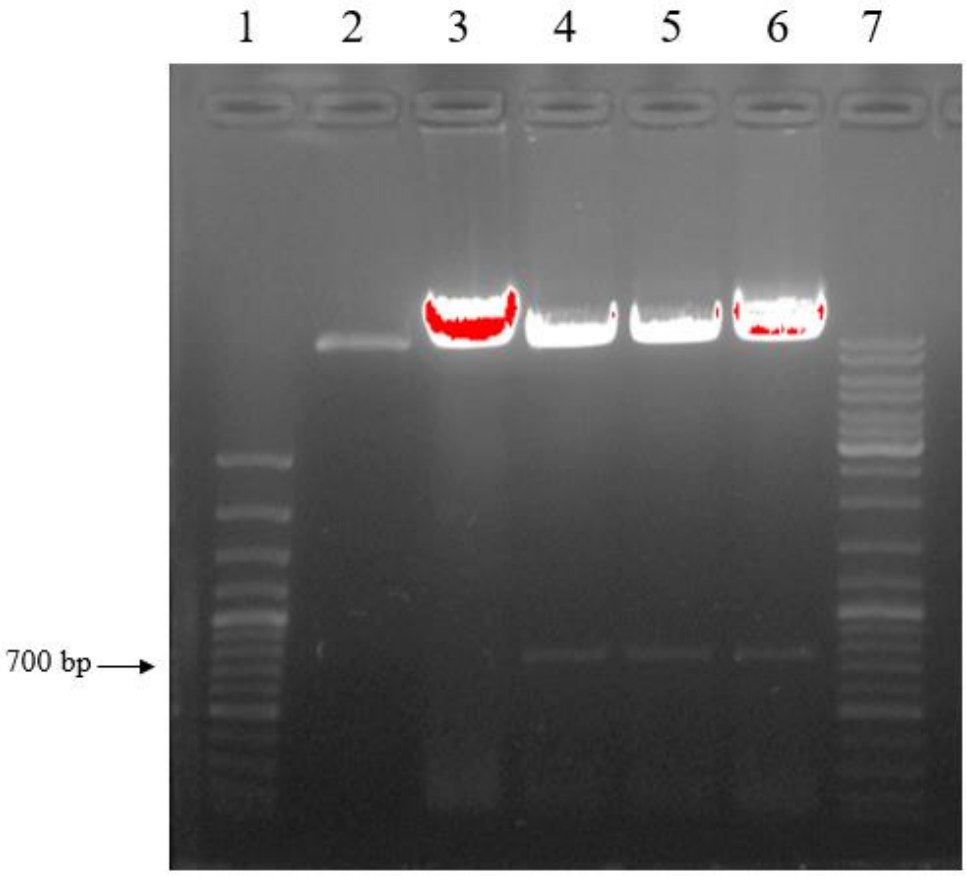

Figure 8. Agarose gel electrophoresis of ClaI and NotI digested pCVA9 and pCVA9-eGFP plasmids. Lane 1: GeneRuler 100 bp Plus DNA Ladder. Lane 2: ClaI-digested pCVA9 (single 7500 bp band). Lanes 3-5: NotI-digested pCVA9-eGFP plasmids 6, 7, and 11 (bands at 700 bp and 7500 bp). Lane 6: GeneRuler DNA Ladder Mix.

For the pCMV-T7Pol, ten different samples were digested individually with the restriction enzymes Hind III and Kpnl resulted, as shown in Figure 9, in one band corresponding to the 10297 bp plasmid when digested with Hind III, while digestion with Kpnl is expected to produce two fragments of 2504 bp and 7793 bp.

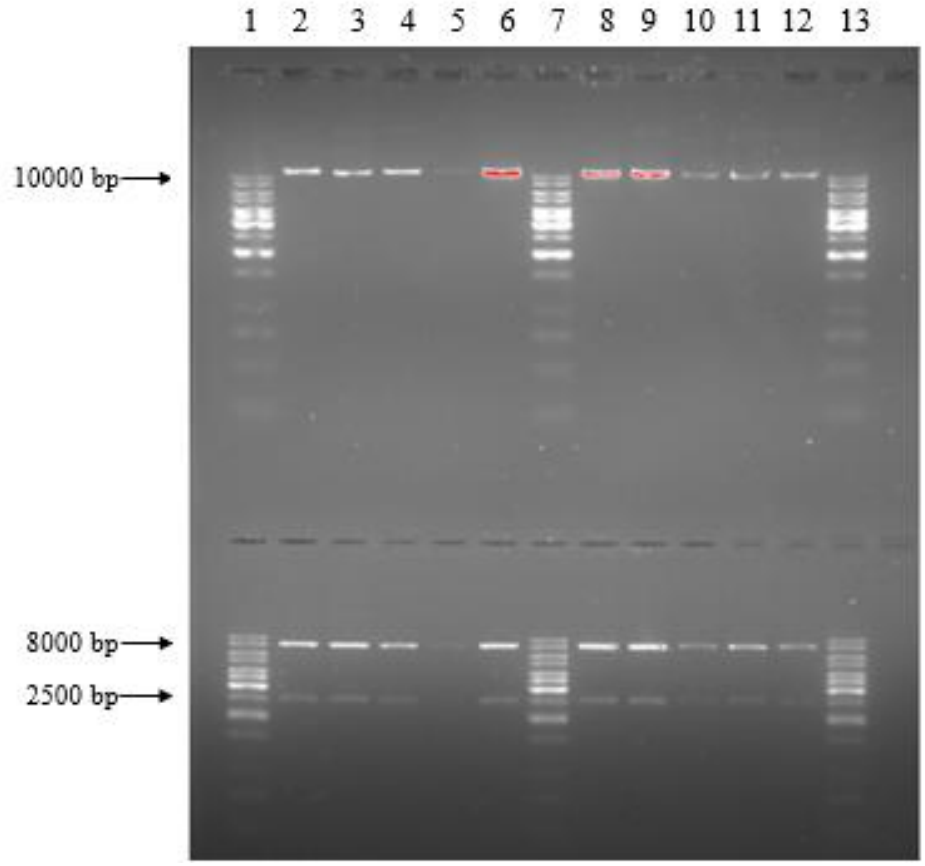

Figure 9. Agarose gel electrophoresis of HindIII and KpnI digested pCMV-T7Pol samples. The first row shows samples digested by HindIII, and the second row shows KpnI digestion products. Lanes 1, 7, and 13: 1 kb GeneRuler DNA ladder. Lanes 2-6: HindIII-digested samples (single band ~1000 bp). Lanes 8-12: KpnI-digested samples (bands at 2504 bp and 7793 bp).

With the exception of lane 5 all of the remaining samples consistently displayed the expected banding pattern, However, only the cell and plasmid stocks corresponding to T7-1 (lane 2) were used in further experiments.

For pCMV/T7-T7pol, a sample was digested individually with the restriction enzymes EcoRI, HindIII as well as additional digestion with HindIII and BamHI combined. Although samples digested with EcoRI showed three bands on the gel, digestion using HindIII resulted as expected

in a single band corresponding to the 8400bp plasmid. Moreover, double digestion with Hind III and BamHI also confirmed the plasmid construct as this digestion is expected to produce two fragments of 3000bp and 5400 bp, as shown in Figure 10.

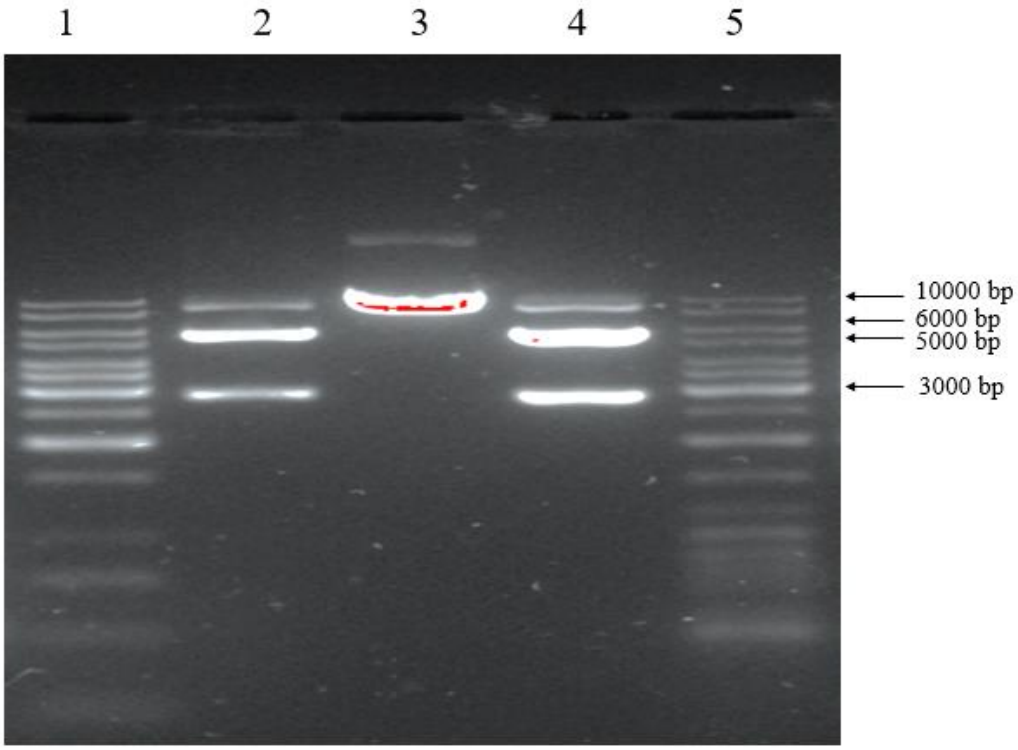

Figure 10. Agarose gel electrophoresis of EcoRI, HindIII, and HindIII + BamHI digested pCMV/T7-T7pol plasmid. Lanes 1 and 5: 1 kb GeneRuler DNA ladder. Lane 2: EcoRI-digested pCMV/T7-T7pol (8400 bp). Lane 3: HindIII-digested pCMV/T7-T7pol. Lane 4: HindIII + BamHI double digestion.

3.2 Large-scale isolation

Large-scale isolation of the plasmid containing the desired MuA gene is an essential step in this study as approximately 1000 µg of purified MuA plasmid DNA is needed to generate the necessary MuA-transposon mutagenesis complex setup. Therefore, three Maxiprep kits were utilized in this project. The standard protocol provided by the manufacturer was initially followed, but due to suboptimal results, other attempts were made with slight modifications. These modifications aimed to enhance the efficiency of the purification process.

The initial purification using the QIAGEN® Plasmid Plus Maxi Kit resulted in a concentration of 88.428 ng/µl. However, a notable improvement was noticed after using a different vacuum system during the second attempt. This improvement yielded four tubes, each containing 400

µl of purified MuA plasmid DNA, with a concentration of 200.156 ng/µl, resulting in a total of 320 µg of purified MuA plasmid DNA.

Digestion of one of these samples of purified MuA plasmid DNA individually using the following enzymes: NotI, BamHI, BglII (double cutters), and EcoRI, HindIII (single cutters), as well as an additional digestion with EcoRI + HindIII. This resulted as shown in the figure bellow in two fragments of 1200 bp, containing the MuA gene, and 2700 bp, the remaining plasmid backbone, when digested with NotI, BamHI, BglII as well as with EcoRI + HindIII. Conversely, the single cutters produced a single fragment larger than the used ladder (GeneRuler 100 bp Plus DNA Ladder), as the size of the MuA plasmid used in this study is 3897 bp.

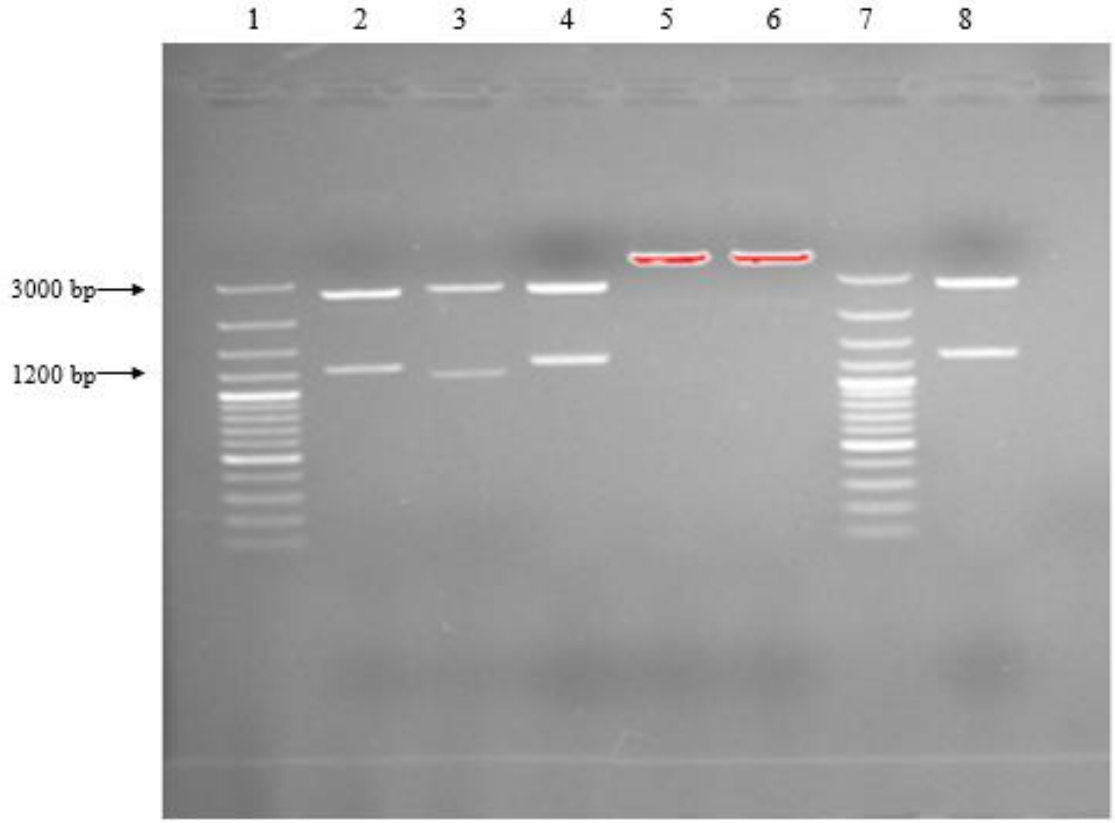

Figure 11. Restriction digestion analysis of purified MuA plasmid DNA with various enzymes. Lanes 1 and 7: GeneRuler 100 bp Plus DNA Ladder, Lane 2: NotI, Lane 3: BamHI, Lane 4: BglII, Lane 5: EcoRI, Lane 6: HindIII and Lane 8: EcoRI + HindIII. Single enzyme digestions show a single fragment (3897 bp), while double digestion shows two fragments (1200 bp and 2700 bp).

Additionally, purification using the Sigma GenElute™ HP Endotoxin-Free Plasmid Maxiprep Kit yielded the lowest concentration among all the different Maxiprep kits used, with a concentration of 18.796 ng/µl.

For the three initial attempts at purifying MuA plasmid DNA with MACHEREY-NAGEL NucleoBond Xtra Maxi Plus kit for transfection-grade plasmid DNA, each attempt yielded four tubes, each containing 500 µl of purified MuA plasmid DNA, with a concentration of 30 ng/µl, 60 ng/µl and 189.801ng/µl respectively. This resulted in a total of 559.603 µg of purified MuA plasmid DNA across the three attempts.

The fourth attempt with the introduced modification as described in the methods and materials section, showed promising results prior to the Isopropanol precipitation step as it resulted in a total of 32 ml of eluted DNA, with a concentration of 17.895 ng/µl thus totaling an amount of 572.64 µg of DNA. However, after doing the Isopropanol precipitation to concentrate, desalt and recover nucleic acids. the total amount of MuA DNA obtained was 150 µg, 4 tubes of 500 µl with a concentration of 75 ng/µl.

As mentioned earlier large-scale isolation of the plasmid containing the desired MuA gene is an essential step as approximately 1000 µg of purified MuA plasmid DNA is needed to generate the necessary MuA-transposon mutagenesis complex setup. Consequently, the total amount of purified MuA DNA obtained across all the different Maxiprep kits used in this study yielded a total amount of 1029.603 µg, this DNA is being further processed in collaboration with Professor Harri Savilahti (Department of Biology, University of Turku, Finland) for further Phenol-chloroform DNA extraction and assembly of transposon complexes.

Table 7. Summary of MuA DNA Plasmid Isolation Results Using Different Maxiprep Kits. Cells shaded in grey indicate attempts excluded from further analysis.

KIT	Concentration (ng/µl)	Amount obtained (µg)
QIAGEN® Plasmid Plus Maxi Kit (1st attempt)	88.428	
QIAGEN® Plasmid Plus Maxi Kit (2nd attempt)	200.156	320, 249
Sigma GenElute™ HP Endotoxin-Free Plasmid Maxiprep Kit.	18.796	
MACHEREY-NAGEL NucleoBond Xtra Maxi Plus kit for transfection-grade plasmid DNA (1st attempt)	30	709.603
MACHEREY-NAGEL NucleoBond Xtra Maxi Plus kit for transfection-grade plasmid DNA (2nd attempt)	60	
MACHEREY-NAGEL NucleoBond Xtra Maxi Plus kit for transfection-grade plasmid DNA (3rd attempt)	189,801	
MACHEREY-NAGEL NucleoBond Xtra Maxi Plus kit for transfection-grade plasmid DNA (4th attempt)	75	
Total amount of purified plasmid DNA (µg)	1029,852	

Following enzymatic digestion and plasmids construct verification by agarose gel electrophoresis, the remaining plasmids, pCVA9, pCVA9-eGFP6, pCVA9-eGFP7, pCVA9-eGFP11, pCMV-T7Pol and pCMV/T7-T7pol, were purified using Sigma's GenElute™ HP Endotoxin-Free Plasmid Maxiprep Kit. The concentration of pCVA9 was measured at 341.527 ng/µl, while pCVA9-eGFP6 exhibited a concentration of 654.007 ng/µl. Plasmids pCVA9-eGFP7, pCVA9-eGFP11, CMV-T7RNAp, and CMV-T7/T7RNAp showed concentrations of 497.239 ng/µl, 176.869 ng/µl, 124.493 ng/µl, and 132.007 ng/µl, respectively.

3.3 Amplification of T7-promoter-tagged full-length CVA9 and CVA9-eGFP genomes

Using the high-fidelity Platinum SuperFi II DNA polymerase enzyme, a forward primer containing the T7 promoter sequence (pCAV9gen1-F) and three different reverse primers (pCAV9gen1-R, CVA9gen1-R_T7-T^{orig}, and CVA9gen1-R_T7-T^{synt}), T7-promoter-tagged PCR amplicons were produced using of pCVA 9 and the three forms of pCVA9-eGFP (pCVA9-eGFP6, pCVA9-eGFP7, and pCVA9-eGFP11) as templates for the long PCR reaction, described in more detail in the section 2.7. A standardized concentration at 10 ng/µl was set for the plasmid templates. The initial amplification reaction used 1 µl of template DNA from which 20 µl of amplified T7-tagged PCR amplicon was produced. Verification of the PCR reaction was done using agarose gel electrophoresis, as shown in Figure 12. Furthermore, 5 µl of each

amplicon was mixed with 1 µl of 6X TriTrack DNA loading dye (Thermo Fisher Scientific) and was loaded into the agarose gel. T7-promoter-tagged CVA9 amplicon shows a band between 6000bp and 8000bp markers, corresponding to the 7500 bp CVA9 genome, while the T7-promoter-tagged CVA9-eGFP(s) amplicons produced bands slightly above the 8000bp marker, consistent with the 8400 bp size of the CVA9-eGFP construct.

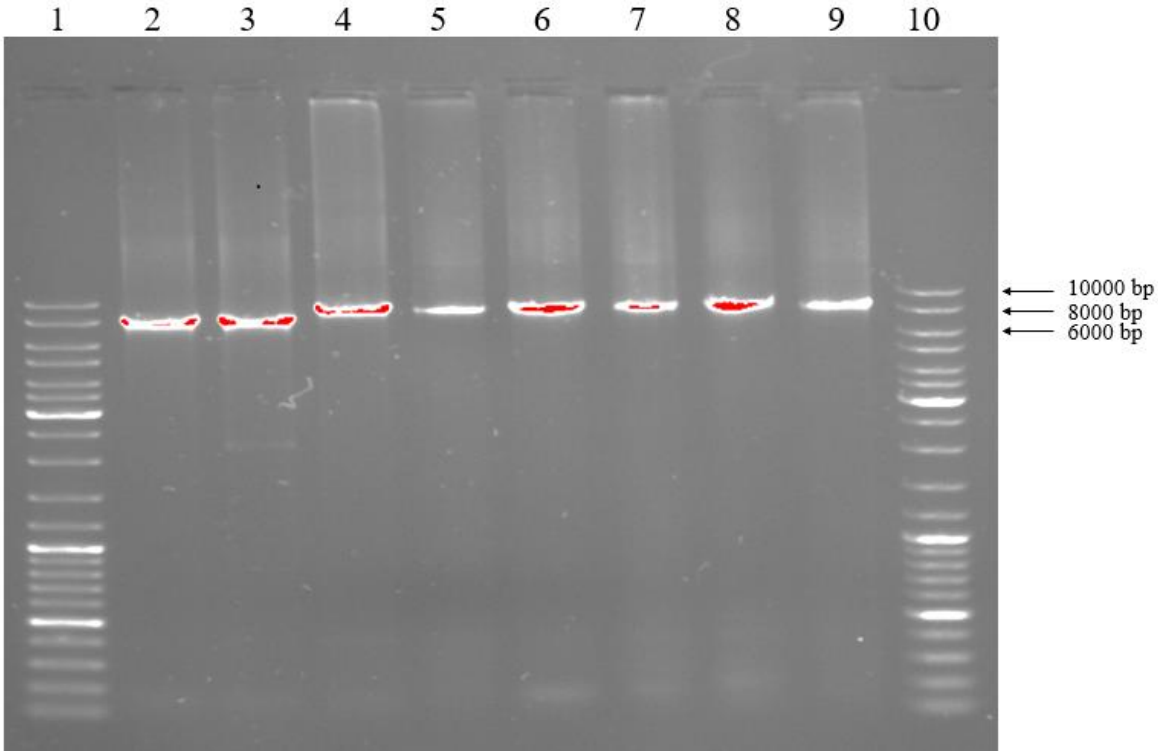

Figure. 12 Agarose gel electrophoresis analysis of T7-promoter-tagged CVA9 and CVA9-eGFP PCR amplicons. Lanes 1 and 10: GeneRuler 1 kb DNA ladder mix, 2 and 3: CVA9 amplicon, 4 and 5: CVA9-eGFP6 amplicon, 6 and 7: CVA9-eGFP7 amplicon, 8 and 9: CVA9-eGFP11 amplicon. The sizes of CVA9 and CVA9-eGFP are 7.5 and 8.4 kb, respectively.

3.4 Transfection efficiency and rescue of infectious coxsackievirus A9 particles

For cell transfection and virus rescue, pCVA9, pCVA9-eGFP(s) and their T7-tagged CVA9-PCR amplicons, were transfected into BHK-21 and T7-BSR cells using Lipofectamine™ 3000 Reagent. Cells were incubated for 24-48 hours to allow infection to proceed. Following incubation, wells were transferred to a fresh medium for endpoint titration via 10-fold dilution. After an additional 24 hours, cells were fixed and stained with a DAPI marker, allowing us to visualize the cell nuclei. EVOS FL AUTO fluorescence microscope was used to examine cells

using both DAPI and GFP modes to assess cell morphology and detect GFP expression, which would indicate viral infection.

Following transfection, two wells containing T7-BSR cells transfected with CVA9-eGFP6 T7-PCR amplicons showed green fluorescence, indicating successful infection. However, green fluorescence was not detected in any of the other wells containing T7-BSR cells or BHK-21.

One of the two wells that showed fluorescent activity was transfected with a CVA9-eGFP6 T7-PCR amplicon amplified using the reverse primer CVA9gen1-R_T7-T^{orig}, while the other well was transfected with a CVA9-eGFP6 T7-PCR amplicon amplified using the reverse primer CVA9gen1-R_T7-T^{synt}.

Although three different plasmid/amplicon concentrations versus Lipofectamine™ 3000 transfection reagent ratios (1:1, 1:3, and 3:1) were tested, both wells that showed green fluorescence were wells with a ratio of 1:1.

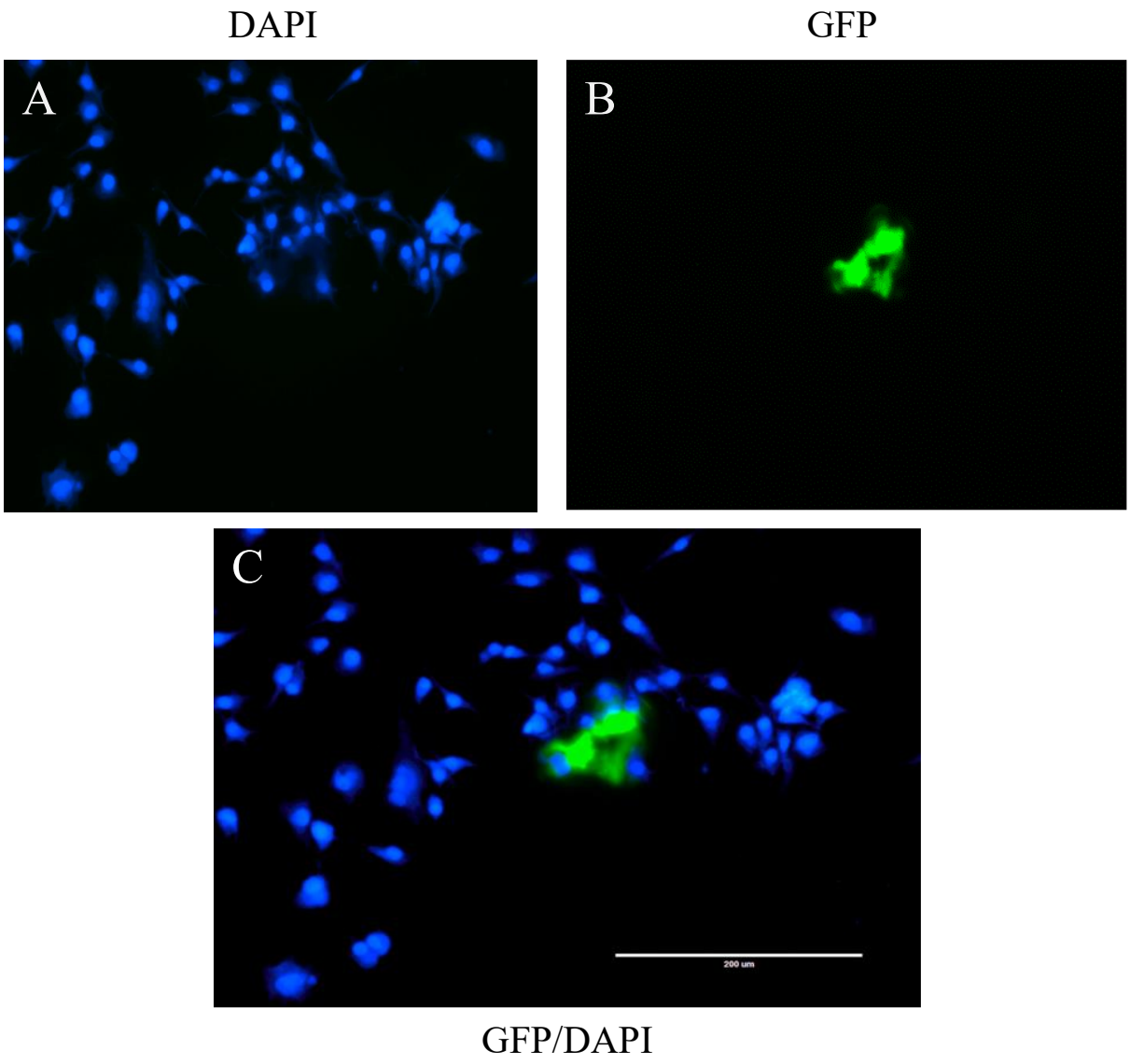

Figure 13. Visualization of CVA9 infectious virus particles in transfected T7-BSR cells. CVA9-eGFP6 T7-tagged PCR amplicons synthesized using the pCAV9gen1-F forward primer and the CVA9gen1-R_T7-T^{orig} reverse primer were transfected into T7-BSR cells. (A) T7-BSR cells stained with DAPI. (B) Green fluorescence indicates GFP production from viral mRNA during replication. (C) Detection of infectious CVA9 particles by green fluorescence.

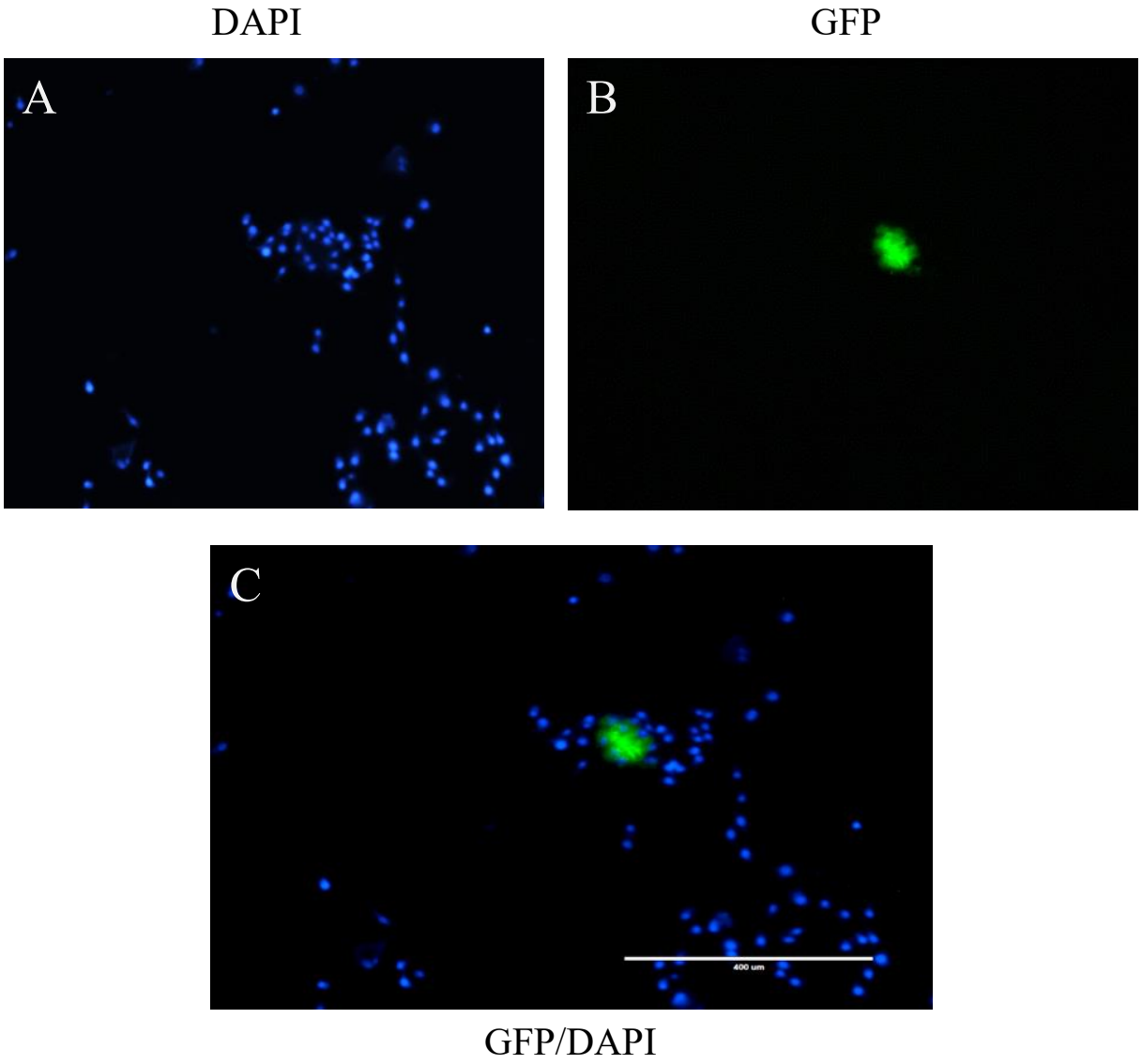

Figure 14 Visualization of CVA9 infectious virus particles in transfected T7-BSR cells. CVA9-eGFP6 T7-tagged PCR amplicons synthesized using the pCAV9gen1-F forward primer and the CVA9gen1-R_T7-T^{synt} reverse primer were transfected into T7-BSR cells. (A) T7-BSR cells stained with DAPI. (B) Green fluorescence indicates GFP production from viral mRNA during replication. (C) Detection of infectious CVA9 particles by green fluorescence.

4 Discussion

This thesis aimed at establishing the foundational components that will allow for the implementation of the Mu transposon system, a transposition-based in vitro insertional mutagenesis strategy, to create a library of mutated coxsackievirus A9 (CVA9-eGFP vector). To achieve this, the proper transposon mutagenesis system had to be produced through isolation, purification, and digestion of MuA plasmid DNA in order to assemble the MuA-transposon complexes. Collaboration with Prof. Harri Savilahti from the Department of Biology at the University of Turku was instrumental in this process. In addition to producing the Mu transposon system, this study investigated the functionality of coxsackievirus A9 sequences through virus rescue. Once mutagenesis takes place, subsequent genome sequencing is used to map the exact location of insertions, allowing us to assess the impacts of these mutations on virus propagation and functionality. The insights gained from this work would ultimately allow for a strategic introduction of additional genetic material to the virus genome without disturbing its replication.

4.1 Production of the MuA-transposon complexes

As mentioned earlier, approximately 1000 µg of purified MuA plasmid DNA is needed to generate the necessary MuA-transposon mutagenesis complex setup. Thus, large-scale isolation of the plasmid containing the desired MuA gene was a significant part of this thesis. Especially as the desired amount of DNA was not successfully isolated initially. Prompting the use of three different maxiprep kits. The kits yielded varying levels of purified DNA. In addition to using different kits following the manufacturer's protocol, slight modifications were also introduced in an attempt to increase the concentration of purified DNA.

Out of the two purification attempts using the QIAGEN® Plasmid Plus Maxi Kit, the second attempt yielded a significantly higher concentration. The concentration from the first attempt was 88.428 ng/µl., while the second attempt yielded 200.156 ng/µl resulting in a total of 320 µg of purified MuA plasmid DNA. This significant improvement is most likely due to implementing a different vacuum system during the second attempt. Therefore, the choice of the vacuum system appears to be important in optimizing DNA yield and purity.

The plasmid DNA purification attempt using the Sigma GenElute™ HP Endotoxin-Free Plasmid Maxiprep Kit, despite following the manufacturer's protocol, resulted in the lowest concentration of MuA plasmid DNA (18.796 ng/µl) compared to the other Maxiprep kits

utilized in this study. It is worth mentioning that all other plasmids, besides the MuA-carrying plasmid, were purified using the Sigma GenElute™ HP Endotoxin-Free Plasmid Maxiprep Kit. However, the concentration obtained for other plasmids proved to be much higher than the one obtained from the MuA plasmid.

The three attempts using the MACHEREY-NAGEL NucleoBond Xtra Maxi Plus kit for transfection-grade plasmid DNA yielded varying concentrations of purified MuA DNA, totaling 559.603 µg. Moreover, the elution step modifications that were introduced in the fourth attempt showed initially a promising impact as they yielded a total of 572.64 µg of DNA before precipitation. However, the Isopropanol precipitation step resulted in a lower final yield compared to the initial concentration obtained after elution with a concentration of 75 ng/µl per tube and a total amount of 150 µg. This discrepancy suggests that further optimization of the precipitation step is necessary to maximize DNA yield in future experiments.

Given the notable discrepancy in yield observed with the MuA plasmid, in comparison to other plasmids used in the study, further optimization of the purification protocol may be needed. To address this, adjustments to lysis conditions or elution parameters can be explored to enhance DNA recovery specifically for the MuA plasmid.

Although large-scale isolation of the plasmid containing the desired MuA gene proved to be more difficult and time-consuming than initially expected requiring the use of several kits and introducing modifications to the manufacturer's protocol, the total amount of purified MuA plasmid DNA across all attempts did reach a total of amount of 1029.603 µg and thus reached the approximately 1000 µg of purified MuA plasmid DNA needed to generate the necessary MuA-transposon mutagenesis complex setup.

4.2 CVA9-eGFP functional validation

The expected band sizes of PCR products were obtained through agarose gel electrophoresis, confirming the successful generation of full-length CVA9 and CVA9-eGFP genomes through the use of high-fidelity Platinum SuperFi II DNA polymerase along with specific T7-promoter-tagged primers.

The transfection results show the efficacy of the T7 RNA polymerase-expressing T7-BSR cell line in virus rescue and the detected green fluorescence in the wells transfected with CVA9-eGFP6 T7-PCR amplicons, produced using the reverse primers CVA9gen1-R_T7-T^{orig} and

CVA9gen1-R_T7-T^{synt}, indicates successful replication of the viral genome. Fluorescence signifies the successful transcription of viral DNA into mRNA via the T7 polymerase enzymes produced by the T7-BSR cells, subsequent translation of this mRNA produces several proteins including the green fluorescent protein (GFP).

On the other hand, the lack of green fluorescence in BHK-21 cells indicates that virus replication and propagation did not take place in these cells. This is because, unlike T7-BSR cells, BHK-21 cells do not produce T7 RNA polymerase, reflecting the importance of this enzyme for viral replication. Although BHK-21 cells served here as a control cell line, the results still emphasize the need for cotransfection with a T7 RNA polymerase expression plasmid to test viral replication in this cell line.

In addition to the importance of having an appropriate cellular environment and the availability of necessary transcriptional machinery, the success with the 1:1 DNA to Lipofectamine™ 3000 reagent ratio further underscores the sensitivity of transfection efficiency and achieving virus rescue.

Moreover, two out of the three used reverse primers, CVA9gen1-R_T7-T^{orig} and CVA9gen1-R_T7-T^{synt}, were successful at producing functional and stable CVA9-eGFP6 T7-PCR amplicons which shows the impact of primer design on the produced viral genome.

4.3 Evolutionary perspectives and significance

For viruses, fitness refers to the capacity of a virus to produce infectious progeny in a given environment (Wargo & Kurath, 2012). Therefore, the use of the Mu transposition system for genome-wide mapping of insertion sites essential for the propagation of coxsackievirus A9 (CVA9) has significant implications for evolutionary biology as this project is ultimately a fitness study assessing the impact of different induced mutations mimicking natural genetic variation effects on virus replication and propagation.

Mapping the genome to identify conserved regions essential for viral propagation provides insights into the genetic components that had to evolve in order to ensure the success of CVA9 as a pathogen. This is because conserved genomic regions reflect the evolutionary constraints that maintain the structural integrity and functionality of the virus. On the other hand, tolerated variation in the other regions indicates genetic flexibility and the virus's adaptability, as both adaptive and neutral variations can lead to the evolution of novel viral strains with different

properties. Such fitness mapping reveals the delicate balance between genetic stability and adaptability that viruses must maintain to thrive in various environments.

As the mutagenesis technique addressed in this study is species-non-specific, it can be applied to other viruses. The identification of conservatively constrained regions across different viruses can be used for comparative genomics purposes. By applying such a mutagenesis technique on other viral genomes, a compression of the findings will allow researchers to study evolutionary relationships and understand how different viruses have adapted to various host environments. Such comparative analyses are fundamental to constructing a comprehensive picture of virus evolution. This is especially important in the case of picornaviruses, as they are a large, genetically heterogeneous group of viruses, exhibiting high mutation rates (Ferrer-Orta et al., 2015), and with several members yet to be classified (Zell et al., 2017; https://www.picornaviridae.com).

4.4 Future research directions

Several of the originally planned experiments had to be omitted from this thesis project due to time constraints and unforeseen delays. For instance, producing the required amount of purified MuA plasmid DNA for the needed transposon mutagenesis system. Therefore, several avenues remain unexplored. Nevertheless, these should form the basis for future work.

The next step would be the actual mutagenesis of the target virus, utilizing the addressed MuA-mediated transposition-based insertional mutagenesis strategy and the functionally verified T7-PCR amplicon of the CVA9-eGFP6 plasmid, the use of a CVA9 genomic amplicon tagged with a T7 promoter will be useful as viral replication will be driven by T7 RNA polymerase binding to the T7 promoter to synthesize the viral RNA and eventually produce functional virus particles. Consequently, the mutagenesis will take place in the T7-BSR cells as they endogenously produce the T7RNA polymerase. Moreover, Future works could also test the mutagenesis in other cells that don't have an intrinsic T7 RNA polymerase activity, but instead through the co-transfection of these cells with the T7-PCR amplicon and a T7 RNA polymerase-producing plasmids, such as pCMV-T7Pol and pCMV/T7-T7pol.

The subsequent step would be the genome sequencing of the viable virus mutants to identify the sites that tolerated the insertional mutations. Through this genome-wide mapping, we can determine crucial genomic regions for CVA9 propagation. Such a functional map of the virus's genome will allow us to determine the non-essential regions where additional genetic material

could be inserted without affecting the virus's ability to replicate, which is critical for developing CVA9 as a vector for oncolytic virotherapy.

Additionally, future studies could adopt a more evolutionary-oriented approach by conducting, for instance, a fitness comparative analysis of the different produced mutants. This will allow us to not only identify the regions that tolerated the mutations but also if there are fitness differences between the viable mutants. Identifying mutants with lower fitness can provide insights into the potential vulnerabilities that can be targeted for therapeutic purposes, which is especially important for picornaviruses, considering the limited availability of antiviral drugs and vaccines for this group of viruses (Lanko et al., 2021).

The knowledge gained from studying CVA9 can be applied to other members of the *Picornaviridae* family since the Mu transposition mutagenesis is a universal technique. Such a broad investigation of picornaviruses can reveal common mechanisms and unique features, contributing to a more comprehensive understanding of this diverse virus family. In addition, studying other picornaviruses could be useful, knowing that many of them, especially within the enterovirus genus, show oncolytic properties (Ylä-Pelto et al., 2016).

Furthermore, future endeavors also aim at utilizing a Mu transposition-based insertional mutagenesis strategy to generate genetically modified oncolytic enteroviruses with enhanced cell tropism, reduced pathogenicity, greater replication capacity and ability to produce cytotoxic proteins in cancer cells by introducing additional genetic material into their genomes.

5 Conclusions

This thesis serves as a foundational framework for employing the Mu transposon system, a transposition-based in vitro insertional mutagenesis strategy, in producing a library of mutated coxsackievirus A9 (CVA9-eGFP vector), through producing the transposon mutagenesis system and investigating the functionality of CVA9 sequences through virus rescue.

Several purification protocols were explored and yielded varying concentrations of purified MuA plasmid DNA. Although the process was more time-consuming than expected, protocol and equipment manipulations yielded satisfactory results that meet the required amount for the assembly of transposon complexes.

The functionality of a viral genome construct was validated by amplifying full-length CVA9-eGFP genome, efficient transfection and virus rescue in T7-BSR cells. viral replication and protein expression were confirmed by the detected green fluorescence. This work highlights the importance of T7 RNA polymerase, the critical role of primer design and transfection conditions.

The research holds evolutionary perspectives and significance. This is because, the implementation of a random insertional mutagenesis technique to identify conservatively constrained regions within the CVA9 genome, offers insights into the genetic makeup and adaptive landscape of the virus enriching our understanding of viral fitness and the evolutionary pressures that shape viral genomes.

In addition to the actual mutagenesis of the target virus and genome sequencing of viable virus mutants, future directions of the project include a comparative analysis of the viable mutant's fitness, as well as, extending the methodologies addressed in this thesis to other picornaviruses and their use in oncolytic virotherapy.

The thesis paves the way for future research on picornavirus biology and the development of genetically altered viruses for therapeutic use. regardless of the obstacles and the unexplored plans, the findings contribute to our understanding of viral evolution and are promising for future advancements.

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