

Chapter 6

Polymerase Mechanism-Based Method of Viral Attenuation

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Abstract

Vaccines remain the most effective way of preventing infection and spread of infectious diseases. These prophylactics have been used for centuries but still to this day only three main design strategies exist: (1) live attenuated virus (LAV) vaccines, (2) killed or inactivated virus vaccines, (3) and subunit vaccines of the three, the most efficacious vaccines remain LAVs. LAVs replicate in relevant tissues, elicit strong cellular and humoral responses, and often confer lifelong immunity. While this vaccine strategy has produced the majority of successful vaccines in use today, there are also important safety concerns to consider with this approach. In the past, the development of LAVs has been empirical. Blind passage of viruses in various cell types results in the accumulation of multiple attenuating mutations leaving the molecular mechanisms of attenuation unknown. Also, due to the high error rate of RNA viruses and selective pressures of the host environment, these LAVs, derived from such viruses, can potentially revert back to wild-type virulence. This not only puts the vaccinee at risk, but if shed can put those that are unvaccinated at risk as well. While these vaccines have been successful there still remains a need for a rational design strategy by which to create additional LAVs.

One approach for rational vaccine design involves increasing the fidelity of the viral RdRp. Increased fidelity decreases the viral mutational frequency thereby reducing the genetic variation the virus needs in order to evade the host imposed bottlenecks to infection. While polymerase mutants exist which decrease viral mutation frequency the mutations are not in conserved regions of the polymerase, which doesn't lend itself toward using a common mutant approach toward developing a universal vaccine strategy for all RNA viruses. We have identified a conserved lysine residue in the active site of the PV RdRp that acts as a general acid during nucleotide incorporation. Mutation from a lysine to an arginine results in a high fidelity polymerase that replicates slowly thus creating an attenuated virus that is genetically stable and less likely to revert to a wild-type phenotype. This chapter provides detailed methods in which to identify the conserved lysine residue and evaluating fidelity and attenuation in cell culture (in vitro) and in the PV transgenic murine model (in vivo).

Key words RNA virus, RNA-dependent RNA polymerase, Polymerase fidelity, Live-attenuated virus, Vaccine, Attenuation, Poliovirus, Sequence homology

1 Introduction

LAVs remain the most effective strategy for vaccine design [1, 2]. However, in the past developments of these vaccines have been empirical. Blind passage of viruses in different cell types results in the accumulation of multiple attenuating mutations leaving the

molecular mechanisms of attenuation unknown. Due to the high error rate of RNA viruses and the selective pressures of the host environment, these LAVs can potentially revert back to wild-type virulence. This not only puts the vaccinee at risk, but if shed can put those that are unvaccinated at risk as well. LAV vaccines have been created against a number of RNA viruses, such as poliomyelitis, measles, mumps, rabies, rubella, yellow fever and influenza. While these vaccines have been successful there still remains a need for a rational design strategy in which to create additional LAVs. It has been shown that by altering fidelity, the rate and speed at which the polymerase incorporates mutations, leads to viral attenuation [3–8].

RNA viruses are defined by high mutation rates, high yields, and short replication times. These viruses have an average mutation rate of 10^{-3} to 10^{-5} mutations per genome replication event [4]. As a result, RNA viruses do not replicate as a single sequence but as a “cloud” of mutant genomes, which have been dubbed *quasispecies* [9–12]. Although a high mutation rate can lead to deleterious changes in the genome, genetic diversity in RNA virus populations appears to be critical for fitness and survival and likely contributes to pathogenesis. In a heterogeneous pathogen population, some variants are able to infect primary tissues and bypass host-imposed bottlenecks. From here the remaining variants can replicate into another heterogeneous population where some are once again able to bypass another layer of bottlenecks and perform secondary infection in other tissues thus demonstrating that a heterogeneous population, or quasispecies, can be beneficial to the pathogen. This adaptability poses a unique challenge, for example, when it comes to developing antiviral drugs and vaccines.

RNA virus populations are heterogeneous due to error-prone replication by the viral RNA-dependent RNA polymerase (RdRp) which influences quasispecies evolution. This adaptability benefits the pathogen sometimes at the cost of the host. Currently, error-prone replication is known to happen in all RNA viruses that infect both plants and animals. It is also known that this error is due to rapid generation of variants and the fidelity of the viral RdRp [13–15].

In order to study the effect of polymerase mutants on RNA virus heterogeneity, we turn to a model RNA virus, poliovirus. Poliovirus (PV) belongs to the family *Picornaviridae*. This family consists of non-enveloped, positive single strand genomes many of which are important human and animal pathogens. The PV genome can be divided into three parts, the 5'-untranslated region (5'-UTR), a single open reading frame (ORF), and the polyadenylated 3'-untranslated region (3' UTR). Upon entry into the cell, the mRNA is translated as a polyprotein of approximately 3000 amino acids and can be divided into three functionally different regions: P1, P2, and P3. The polyprotein is cleaved cotranslationally and posttranslationally by viral proteases 2A^{pro} and 3C^{pro} into 11 proteins. The PV RdRP is found in the P3 region and is termed 3Dpol.

1.1 Identification of the Conserved Lysine

The RdRp is one out of four categories of polymerases and its crystal structure shows a close evolutionary relationship not only to other RdRps but also to that of DNA-dependent DNA-polymerases (DdDps), DNA-dependent RNA-polymerases (DdRps), and RNA-dependent DNA-polymerases (RdDps) also known as reverse transcriptases (RTs). All resemble a cupped right-handed structure consisting of the thumb, fingers, and palm subdomains [16, 17]. The palm is where the active site of the polymerase lies and consists of four conserved structural motifs A–D [16]. A fifth and sixth motif, E and F, exists in the RNA-dependent polymerases but not the DNA-dependent polymerases [16]. The latter motifs are not in the active site but line this region. RdRps are error prone but they are as faithful as DNA polymerases that lack proofreading exonucleases [18]. The absence of a repair mechanism in the PV genome is what leads to an enhanced rate of mutation during viral replication.

Nucleic acid polymerases use a two-metal-ion mechanism for nucleotidyl transfer [19]. In this mechanism, two magnesium ions are used to organize the reactants. Recently, the chemical mechanism of nucleotidyl transfer has been expanded to include a general acid, which protonates the pyrophosphate leaving group of the NTP substrate and enhance the efficiency of nucleotidyl transfer [20, 21]. The general acid of PV RdRp is Lys359, located in motif D, which is conserved throughout all RdRps and RTs. Importantly, an orthologous residue at this site is known or predicted in RNA viruses for which rational design of vaccines would greatly benefit.

1.2 In Vitro and In Vivo Biological Analysis

In order to determine what effect biochemical changes have on the multiplication of the virus in cell culture we created a PV genome encoding the arginine mutation in the PV subgenomic replicon (pRLucRA) and viral cDNA (pMoVRA). Quantification of virus by plaque assay provides insight into fitness of the viral population. The subgenomic replicon permits indirect evaluation of RNA synthesis by measurement of luciferase activity. Analysis of RNA replication in the absence of virus production can provide insight on whether RNA replication is the rate-limiting step for virus production.

The characteristics of live-virus multiplication and their plaque phenotype can predict whether the virus will be attenuated in the mouse model. However, viral quantification by plaque forming units (pfu) selects variants based on phenotype and therefore can be an unreliable measure of viruses present due to phenotypic differences between viral strains. In addition to pfu, quantifying virus based on genomes accounts for total viral particles produced. This is a more accurate measurement of total viruses in the population and the number of genomes produced by the polymerase.

While these characteristics predict attenuation, actual confirmation is determined using a mouse transgenic for the PV receptor. In this system, wild-type (WT) PV is generally lethal. At the highest dose, the mutated polymerase (lysine to arginine) failed to

cause disease in the mice. To determine if the mutant virus replicated, mice surviving the initial infection were challenged with a lethal dose of WT PV. We can conclude from the mice that survive this challenge, the mutant is replication competent and elicits an immune response sufficient enough to protect against a lethal dose of WT PV [3].

Using PV as our model we have developed a rational design for polymerase-based mechanism of attenuation. By altering the nature of the general acid lysine residue to an arginine, we have shown that we maintain the ability to tune RdRp speed and fidelity creating a viral RdRp that is slower and more faithful than the WT enzyme. This results in an attenuated virus with a restricted viral quasispecies that fails to cause disease, yet elicits a protective immune response. This approach has the ability to be applied to any RNA virus given the conserved nature of the motif D lysine residue.

2 Materials

2.1 Identification of the Conserved Lysine

Table 1 is an alignment of residues found in motif D of the RdRp for positive and negative strand RNA virus families. Numbers indicate the position from the first amino acid of motif D in the RdRp domain. The conserved lysine residue is shown in boldfaced type. Other conserved residues are underlined. All sequences were obtained from the NCBI Database. Sequences were aligned using ClustalW2 and based upon alignments previously published [22].

2.2 Site-Directed Mutagenesis of pMoVRA and pRLucRA by Overlap PCR/Cloning Components

1. Poliovirus Mahoney cDNA, pMoVRA, and subgenomic replicon pRLucRA [23].
2. External primers, diluted to 5 μ M concentrations.
 - (a) Forward: PV-3D-BglII-for (5'-TAG AGG ATC CAG ATC TTG GAT GCC A-3').
 - (b) Reverse: PV-3D-EcoRI-ApaI-polyA-rev (5'-CGC TCA ATG AAT TCG GGC CCT TTT TTT TTT TTT TTT TCT CC-3').
3. Internal primers, diluted to 5 μ M concentrations.
 - (a) Forward: PV-3D-K359R-for (5'-ATG ACT CCA GCT GAC CGTTCA GCT ACA TTT GAA ACA-3').
 - (b) Reverse: PV-3D-K359R-rev (5'-TGT TTC AAA TGT AGC TGA ACG GTC AGC TGG AGT CAT-3').
4. T₁₀E_{0.1} Buffer: 10 mM Tris-HCl, pH 8.0, 0.1 mM ethylene diamine tetraacetic acid [EDTA], pH 8.0.
5. NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific).

Table 1**Motif D sequence alignment across positive and negative strand RNA virus families**

Class	Virus family	Species	Motif D
+ssRNA	Picornaviridae	Poliovirus	15 <u>DY</u> GLTMTPADKSA
		Coxsackievirus B3	15 <u>GY</u> GLIMTPADKGE
		Enterovirus A	15 <u>EY</u> GLTMTPADKSP
		Enterovirus 71	15 <u>EY</u> GLTMTPADKSP
		Human Rhinovirus A	15 <u>KY</u> GLTITPADKSD
		Human Rhinovirus B	15 <u>NY</u> GLTITPPDKSE
		Human Rhinovirus C	15 <u>KY</u> GLTITPADKSD
		Caliciviridae	Norovirus
	Flaviviridae	Dengue 1	10 TALNDMGKVRKDI
		Dengue 2	10 TALNDMGKIRKDI
		Dengue 3	10 LALNDMGKVRKDI
		Dengue 4	10 LFLNDMGKVRKDI
		West Nile Hepatitis C	10 HFLNAMSKVRKDI 21 RYSAPPGDPPKPE
-ssRNA	Togaviridae	Chikungunya	10 RCATWMNMEVKII
		Eastern equine encephalitis	10 RCATWLNMEVKII
		Venezuelan equine encephalitis	10 RCATWLNMEVKII
		Western equine encephalitis	10 RCATWLNMEVKII
		Sindbis	10 RCATWLNMEVKII
	Coronaviridae	SARS	22 YQNNVFMSEAKCW
	Paramyxoviridae	Nipah	59 YD <u>GA</u> VLVLSQALKSM
	Filoviridae	Ebola	59 LN <u>GI</u> QLPQSLKTA
	Orthomyxoviridae	Influenza A	20 LVGINM.TKKKSY
		Influenza B	20 LL <u>GI</u> NM.SKKKSY
Influenza C		20 LI <u>GI</u> NM.SLEKSY	

Numbers indicate position from first amino acid in motif D of RNA-dependent RNA polymerase. Conserved residues are shown in *boldfaced* type. Residues conserved within a virus group are *underlined*

6. Deep Vent DNA polymerase 2000 U/mL (New England BioLabs).
7. 3 mM dNTP mix: 100 mM dATP, 100 mM dGTP, 100 mM dTTP, and 100 mM dCTP. This solution is prepared by combining 300 μ L of each NTP and bringing the volume up to 10 mL with ultrapure water and can be aliquoted and stored indefinitely at 20 °C.
8. 100 mM magnesium sulfate [MgSO₄] solution (supplied with Deep Vent).
9. 10 \times ThermoPol reaction buffer (supplied with Deep Vent).
10. 3 M sodium acetate [NaOAc], pH 5.2: adjust pH with glacial acetic acid.

11. Absolute ethanol.
12. 70 % ethanol solution: 70 % EtOH, 30 % ultrapure water.
13. Omnipur Agarose (Millipore/Calbiochem).
14. 0.5× TBE electrophoresis running buffer: 33 mM Tris-HCl, 40 mM boric acid, 1 mM EDTA, pH 8.0, 0.25 µg/mL ethidium bromide [EtBr].
15. Electrophoresis chamber and power source.
16. 5× bromophenol blue [BPB]: 0.05 % bromophenol blue, 50 % glycerol in T₁₀E_{0.1} buffer.
17. *Bgl*III, *Eco*RI, *Apa*I and *Pst*I restriction enzymes.
18. Shrimp alkaline phosphatase [SAP], 1000 units.
19. QIAEX II Gel extraction kit (Qiagen).
20. Spin-X Plastic Centrifuge Tube Filters (Corning/Costar).
21. T4 DNA ligase, 1 unit/µL.
22. 5× T4 DNA ligase buffer.
23. SURE competent cells (Stratagene).
24. NZCYM broth, powder (Amresco).
25. 100 mg/mL ampicillin solution: 2 g ampicillin in 20 mL ultrapure water.
26. 2 L Erlenmeyer flask.
27. 2 % agar plates prepared with NZCYM medium with 50 µg/mL ampicillin.
28. Qiagen Plasmid Midiprep Kit (Qiagen).

2.3 In Vitro Biological Analysis of Polymerase Mutation Components

2.3.1 Tissue Culture Components

1. Sterile 100 mm polystyrene tissue culture dishes.
2. HeLa S3 cells from American Type Culture Collection (American Type Culture Collection [ATCC] no. CCL-2.2).
3. Complete medium: DMEM/F12, 10 % fetal bovine serum [FBS], 100 U/mL penicillin, 100 U/mL streptomycin.
4. 1× trypsin-EDTA solution.
5. Rapid-Flow Sterile 500 mL bottle top filter with 75 mm PES membrane, 0.22 µm pore size, and 45 mm blue neck, (Thermo Scientific/Nalgene).
6. Autoclaved 1× Phosphate buffered saline [PBS]: Make a 10× PBS solution 1.37 M sodium chloride, 27 mM potassium chloride, 100 mM disodium phosphate [Na₂HPO₄], and 20 mM monopotassium phosphate [KH₂PO₄], pH 7.4. Sterilize the 10× solution using a bottle top filter. Dilute to 1× using ultrapure water and autoclave for 30 min.

2.3.2 *cDNA Linearization
and In Vitro T7
Transcription Reaction
Components*

RNA is easily degraded by RNases, which are ubiquitous in the laboratory. They are found in the air, your skin or on anything touched by bare hands. Prior to beginning in vitro transcription, take care to designate an RNase-free area of the laboratory. Wipe down surfaces and any tools (pipets, beakers, bottles, instruments, etc.) with RNase AWAY. Rinse very well with hot water and spray down with 70 % ethanol solution. Always wear gloves and spray hands with 70 % EtOH before starting any procedure. Purchase chemicals and keep them in a separate area only to be used for making RNase-free solutions. Once RNA is made it can be stored at -80°C until ready for use. RNA made for making virus can be stored for up to 1 week. RNA made for use in luciferase assays must be used the following day.

1. RNase AWAY (Molecular BioProducts).
2. Mutant plasmid.
3. *Apa*I restriction enzyme.
4. QIAEX II Gel extraction kit (Qiagen).
5. Spin-X Plastic Centrifuge Tube Filters (Corning/Costar).
6. T₁₀E_{0.1} Buffer.
7. Ultrapure water.
8. 1 M Hepes, pH 7.5.
9. 320 mM magnesium acetate [MgAcetate].
10. 400 mM dithiothreitol [DTT].
11. 20 mM spermidine.
12. 160 mM NTPs: 40 mM ATP, 40 mM, CTP, 40 mM GTP, and 40 mM UTP.
13. T7 RNA polymerase [RNAP], 0.5 mg/mL.
14. RQ1 RNase-free DNase, 1000 units (Promega).
15. Autoclaved 0.65 mL microcentrifuge tubes.
16. Agarose.
17. RNase-free 0.5× TBE with 0.25 µg/mL ethidium bromide [EtBr].
18. RNeasy Mini Kit (Qiagen).
19. NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific).

2.3.3 *Luciferase Assay
Components*

1. Complete medium.
2. In vitro transcribed RNA made from mutant pRLucRA.
3. Autoclaved 1.7 mL microcentrifuge tubes.
4. VWR Signature Disposable Electroporation Cuvettes, 2 mm (VWR).

5. Bio-Rad Gene Pulser Generator Model 1652076 (Bio-Rad Laboratories).
6. Bio-Rad Capacitance Extender Model 1652087 (Bio-Rad Laboratories).
7. 12 × 75 mm disposable glass borosilicate tubes (VWR).
8. Luciferase assay system: includes luciferase assay substrate, luciferase assay buffer, and 5 × cell culture lysis reagent [CCLR] (Promega).
9. Junior LB 9509 Portable Tube Luminometer (Berthold Technologies).

2.3.4 Poliovirus Stock Components

1. HeLa S3 cells (*see* [Tissue Culture Components](#)).
2. Complete medium (*see* [Tissue Culture Components](#)).
3. In vitro transcribed RNA made from mutant pMoVRA.
4. VWR Signature Disposable Electroporation Cuvettes, 2 mm (VWR).
5. Bio-Rad Gene Pulser Generator Model 1652076 (Bio-Rad Laboratories).
6. Bio-Rad Capacitance Extender Model 1652087 (Bio-Rad Laboratories).

2.3.5 Quantitating Virus and Viral Genomes Components

1. HeLa S3 cells.
2. Complete media (*see* [Tissue Culture Components](#)).
3. 6-well, flat bottom cell culture plates (Corning/costar).
4. 2 × DMEM/F12 complete media: two packages of 1 × DMEM, powder (Gibco), 4.8 g sodium bicarbonate, 20 % FBS, 200 U/mL penicillin, and 200 U/mL streptomycin.
5. Concentrated HCl.
6. Autoclaved, Pyrex Brand 1395 media storage bottle w/screw cap, 1 L (Corning).
7. Rapid-Flow Sterile 500 mL bottle top filter with 75 mm PES membrane, 0.22 μm pore size, and 45 mm blue neck, (Thermo Scientific/Nalgene).
8. Low melt agarose (Omnipur/Calbiochem).
9. 500 mL Erlenmeyer flask.
10. Crystal violet staining solution: 0.1 % crystal violet, 3.7 % formaldehyde, 20 % ethanol made in distilled water.
11. Autoclaved 1.7 mL microcentrifuge tubes.
12. QIAamp Viral RNA mini kit (Qiagen).
13. Qiagen RNeasy Plus Kit (Qiagen).
14. Molecular biology grade β-mercaptoethanol [β-ME].

2.4 *In Vivo* Analysis of Viral Mutation Components

2.4.1 Lethal Dose Fifty (LD_{50})/Paralysis Dose Fifty (PD_{50}) Analysis Components

1. 4–6-week-old outbred (ICR) mice transgenic for the PV receptor (cPVR) [24].
2. Quantitated virus stock, propagated in serum-free media (*see* Quantitating virus and viral genomes components).
3. 5 mL syringes.
4. 27 gauge (G), ½ inch needles.

2.4.2 Protection

1. Immunized mice (survivors from LD_{50}/PD_{50} study).
2. Five times the LD_{50}/PD_{50} [$5 \times PD_{50}$] of wild-type poliovirus.

3 Methods

3.1 Site-Directed Mutagenesis by Overlap Extension PCR

The polymerase gene was amplified using pMoV-3D-BPKN plasmid as a template. This template has silent mutations engineered into the 3Dpol coding sequence. The “naked” viral cDNA, pMoVRA, contains 4 *Pst*I restriction sites and pRLucRA contains 3. A *Pst*I site was engineered into the 3Dpol coding sequence such that when cloned into the “naked” vectors, pMoVRA and pRLucRA, and digested with *Pst*I, positive clones containing the mutated PCR product will have 5 and 4 bands respectively when run on an agarose gel. Clones positive by restriction digest are verified for the presence of the mutation by sequencing.

3.1.1 Round One: Extension PCR

1. PCR reaction A:
 - (a) External forward primer: PV-3D-BglIII-for.
 - (b) Internal reverse primer: PV-3D-K359R-rev.
2. Perform amplification reactions in three separate 100 μ L volumes, final concentration containing: 1 \times Thermopol buffer, 3 mM dNTPs, 0.5 μ M of each primer, 0.5 ng/ μ L of template plasmid pMo-3D-BPKN, and 2 U of Deep Vent Polymerase (Table 2).
3. Cycling conditions consist of a preliminary denaturing step at 95 °C for 4 min followed by a hot start cycle for 4 cycles at 95, 50, and 72 °C each for 1 min and finally, 18–20 cycles of denaturation at 95 °C for 1 min, annealing at 57 °C for 1 min and product extension at 72 °C for 2 min and a final product extension at 72 °C for 10 min.
4. Prepare 2, 1.2 % agarose gels.
5. The rest of product tubes are combined and DNA is precipitated with 100 % ethanol. Add 1/5th volume (60 μ L) 3 M NaOAc, mix well with pipet then add three volumes (1080 μ L) 100 % EtOH and mix well. Freeze mixture on dry ice until liquid is a slow moving “sludge” when inverted. Centrifuge at top speed for 10 min. You will observe a thick white pellet.

Table 2**Round 1–1: extension PCR—reaction A**

Reagent	Volume (μL)			Reaction concentration
10 \times Thermopol reaction buffer	10	10	10	1 \times
100 mM MgSO_4	0	1	2	0/1/2 mM
3 mM dNTPs	10	10	10	0.3 mM
5 μM forward primer: PV-3D-K359R-for	10	10	10	0.5 μM
5 μM reverse primer: PV-3D-EcoRI-ApaI-polyA-rev	10	10	10	0.5 μM
5 ng/ μL pMo-3D-BPKN	10	10	10	0.5 ng/ μL
Deep Vent polymerase	1	1	1	2 U
ddH ₂ O	49	48	47	–
<i>Reaction volume</i>	<i>100</i>	<i>100</i>	<i>100</i>	

Wash the pellet with 70 % EtOH three times. Pipet off all the EtOH and allow pellet to air dry for 5–10 min. Suspend in 10 μL T₁₀E_{0.1}.

6. Mix 10 μL of each PCR reaction with 2 μL 5 \times BPB. Load onto 1.2 % agarose gel and run for 30 min at 200 V.
7. Due to the high concentration of DNA loaded, EtBr in the gel and running buffer you should be able to visualize, without a UV light, a red band where the DNA is. Extract DNA from gel and purify using QIAEX II Gel extraction kit. Suspend product in 50 μL T₁₀E_{0.1}. Quantitate purified PCR product using NanoDrop spectrophotometer.
8. PCR reaction B:
 - (a) External reverse primer: PV-3D-EcoRI-ApaI-polyA-rev.
 - (b) Internal forward primer: PV-3D-K359R-for.
9. Same procedure as for PCR reaction A (Table 3).
10. Repeat concentration and purification a for PCR A.

3.1.2 Round Two: Overlap PCR

1. Set up 3–100 μL reactions as before this time using PCR reactions A and B as templates (Table 4):
 - (a) External forward primer: PV-3D-BglIII-for.
 - (b) External reverse primer: PV-3D-EcoRI-ApaI-polyA-rev.
2. Concentrate and purify PCR product as previously described for PCR products A and B.

3.2 Cloning PCR Fragment into pMoVRA and pRLucRA Vectors

3.2.1 Vector and PCR Fragment Digest

1. Perform 20-fold overdigestion of pMoVRA, pRLucRA vectors and overlap PCR fragment with *Bgl*II and *Apa*I restriction enzymes (*see Note 3*).
2. First, digest cDNA and overlap PCR fragment with *Bgl*II. Digest purified cDNA by adding 2 μ g of DNA to a 1.5 mL tube containing 10 μ L of the appropriate 10 \times restriction buffer with 4 μ L (40 U) of enzyme in a total volume of 100 μ L and incubate according to manufacturer's instructions. We recommend 2–4 h for an incubation time. For the purified overlap PCR product, repeat the same procedure as above, using the entire 50 μ L product in the digest (Table 5).
3. Allow reaction to proceed at 37 $^{\circ}$ C for 2 h.
4. Run a sample of uncut and cut plasmids on 1 % agarose gel to check efficiency of reaction (*see Note 3*).
5. When you have verified that the plasmid has been linearized, purify with QIAEX II gel extraction kit. Follow the kit's protocol for purifying and concentrating DNA from an aqueous solution.
6. Clean up DNA using QIAEX II gel extraction kit. Follow the kit's protocol for purifying and concentrating DNA from an aqueous solution.
7. Suspend silica bead pellet in 50 μ L T₁₀E_{0.1} and incubate at 65 $^{\circ}$ C for 10 min.
8. Quick spin the tube and remove both supernatant and beads and add to Spin-X filter tube. Spin at 800 $\times g$ for 5 min and collect eluted DNA from tube.

Table 3
Round 1–2: extension PCR—reaction B

Reagent	Volume (μ L)			Reaction concentration
10 \times Thermopol reaction buffer	10	10	10	1 \times
100 mM MgSO ₄	0	1	2	0/1/2 mM
3 mM dNTPs	10	10	10	0.3 mM
5 μ M forward primer: PV-3D-K359R-for	10	10	10	0.5 μ M
5 μ M reverse primer: PV-3D-EcoRI-ApaI-polyA-rev	10	10	10	0.5 μ M
5 ng/ μ L pMo-3D-BPKN	10	10	10	0.5 ng/ μ L
Deep Vent polymerase	1	1	1	2 U
ddH ₂ O	49	48	47	–
<i>Reaction volume</i>	<i>100</i>	<i>100</i>	<i>100</i>	

Table 4**Round 2: overlap PCR**

Reagent	Volume (μL)			Reaction concentration
10 \times Thermopol reaction buffer	10	10	10	1 \times
100 mM MgSO_4	0	1	2	0/1/2 mM
3 mM dNTPs	10	10	10	0.3 mM
5 μM forward primer: PV-3D-BglII-for	10	10	10	0.5 μM
5 μM reverse primer: PV-3D-EcoRI-ApaI-polyA-rev	10	10	10	0.5 μM
5 ng/ μL PCR reaction A	10	10	10	0.5 ng/ μL
5 ng/ μL PCR reaction B	10	10	10	0.5 ng/ μL
Deep Vent polymerase	1	1	1	2 U
ddH ₂ O	39	38	37	–
<i>Reaction volume</i>	<i>100</i>	<i>100</i>	<i>100</i>	

Table 5**Vector (pMoVRA and pRLucRA) and insert (overlap PCR fragment) digest**

Reagent	Volume (μL)	Reagent	Volume (μL)
10 \times NEB 3.1 buffer	10	10 \times NEB 3.1 Buffer	10
<i>Bgl</i> II (10 U/ μL)	5	<i>Bgl</i> II (10 U/ μL)	5
2 μg cDNA	–	PCR fragment	50
ddH ₂ O	–	ddH ₂ O	35
<i>Reaction volume</i>	<i>100</i>	<i>Reaction volume</i>	<i>100</i>

9. Digest purified cut cDNA by adding entire 50 μL of cut DNA to a 1.5 mL tube containing 10 μL of the appropriate 10 \times restriction buffer with 1 μL (50 U) of enzyme in a total volume of 100 μL and incubate according to manufacturer's instructions. We recommend 2–4 h for incubation time. For the purified cut overlap PCR product, repeat the same procedure as above (Table 6).
10. Allow reaction to proceed at 25 $^\circ\text{C}$ (about room temperature) for 2 h.
11. After the 2 h incubation with *Apa*I to the cDNA only digest, add 4 μL (4 U) of shrimp alkaline phosphatase to reaction. Incubate at 37 $^\circ\text{C}$ for 4 h to overnight in order to

dephosphorylate the ends of the double cut cDNA. Afterwards heat inactivate at 65 °C for 5 min.

12. Run a sample of the double cut plasmids on 1 % agarose gel to check efficiency of reaction.
13. Clean up both the PCR and cDNA reactions using QIAEX II gel extraction kit. Follow the kit's protocol for purifying and concentrating DNA from an aqueous solution.
14. Suspend silica bead pellet in 50 μL T₁₀E_{0.1} and incubate at 65 °C for 10 min.
15. Quick spin the tube and remove both supernatant and beads and add to SpinX tube. Spin at 800 $\times g$ for 5 min and collect eluted DNA from tube.

3.2.2 Ligation Reaction (See Note 4)

1. With *Bgl*II and *Apa*I digested vectors and PCR fragment set up the ligation reaction as follows: Add 50 ng of double cut cDNA (vector), 50 ng of double-cut overlap PCR product (insert), to a 0.65 mL microcentrifuge tube containing 6 μL of 5 \times T4 DNA ligase buffer and 1 μL (1 U) of T4 ligase in a total volume of 30 μL and incubate reaction at 15 °C for 30 min (Table 7).
2. Run 15 μL on a 1 % gel to check for successful ligation and transform 10 μL into 100 μL SURE cells. Plate on 50 $\mu\text{g}/\text{mL}$ ampicillin agar plates and incubate at 30 °C overnight.
3. pMoVRA and pRLucRA are both low copy plasmids. To screen colonies, grow in 500 mL culture with 50 $\mu\text{g}/\text{mL}$ ampicillin at 30 °C to an OD₆₀₀ of 1.0. Purify 1 mL of culture and screen plasmid by *Pst*I restriction digest. Harvest cells and purify plasmid using Qiagen Midi Prep Kit.
4. Successful plasmids should be labeled as pMoV/pRLuc-PV-3D-BPKN-K359R.

Table 6

Cut vector (pMoVRA and pRLucRA) and cut insert (overlap PCR fragment) digest

Reagent	Volume (μL)	Reagent	Volume (μL)
10 \times NEB cut smart <i>buffer</i>	10	10 \times NEB Cut Smart Buffer	10
<i>Apa</i> I (50 U/ μL)	1	<i>Apa</i> I (50 U/ μL)	1
<i>Bgl</i> II digested cDNA	50	<i>Bgl</i> II digested overlap PCR fragment	50
SAP (1 U/ μL)	1	–	–
ddH ₂ O	35	ddH ₂ O	35
<i>Reaction volume</i>	100	<i>Reaction volume</i>	100

3.3 *In Vitro* Biological Analysis of Polymerase Mutation

3.3.1 *cDNA Linearization and In Vitro T7 Transcription Reaction*

1. pMoV-3D-K359R and pRLuc-3D-K359R plasmids are first linearized with restriction enzyme *ApaI*.
2. Digest purified cDNA by adding 5 µg of mutant plasmid to a 1.5 mL tube containing 10 µL of the appropriate 10× restriction buffer with 2.5 µL (50 U) of enzyme in a total volume of 100 µL and incubate according to manufacturer's instructions. We recommend 2–4 h for incubation time (Table 8).
3. Run a sample of uncut and cut plasmids on 1 % agarose gel to check efficiency of reaction (*see Note 3*).
4. When you have verified that the plasmid has been linearized, purify with QIAEX II gel extraction kit. Follow the kit's protocol for purifying and concentrating DNA from an aqueous solution.
5. Suspend silica bead pellet in 50 µL T₁₀E_{0.1} and incubate at 65 °C for 10 min.
6. Quick spin the tube and remove both supernatant and beads and add to Spin-X tube. Spin at 800×*g* for 5 min and collect eluted DNA from tube.
7. Add H₂O first; subtract the DNA volume from 2.5 µL to get the volume of H₂O to be added.
8. Next add the following in this order to a total volume of 20 µL, final concentration containing: 350 mM HEPES, 32 mM MgAcetate, 40 mM DTT, 2 mM spermidine, 28 mM NTPs, 0.5 µg linearized cDNA, and 0.5 µg T7 RNAP into an autoclaved 0.6 mL microcentrifuge tube (Table 9).
9. Pre-incubate the reaction mix at 37 °C for 5 min prior to adding T7 RNAP.
10. Add T7 RNAP.
11. Incubate reaction at 37 °C. After 30 min, check reaction for cloudy, white precipitate, which is magnesium pyrophosphate forming, to ensure the reaction is progressing. Allow reaction

Table 7
Vector and insert ligation reaction

Reagent	Volume (µL)
5× T4 DNA ligase buffer	6
5 ng/µL vector	10
5 ng/µL insert	10
T4 Ligase (1 U/µL)	1
ddH ₂ O	3
<i>Reaction volume</i>	<i>30</i>

Table 8
Linearization of cDNA

Reagent	Volume (μL)
10 \times NEB cut smart buffer	10
<i>Apa</i> I (50 U/ μL)	2.5
5 μg cDNA	–
ddH ₂ O	–
<i>Reaction volume</i>	100

to incubate for 4–5 h, spin the reaction for 2 min to pellet out the magnesium pyrophosphate.

12. Transfer supernatant to a new tube and then add 2 μL of RQ1 DNase (2 U) and incubate for 30 min at 37 °C.
13. Clean up RNA using Qiagen RNeasy Mini Kit following manufacturer's instructions for RNA cleanup.
14. Measure concentration of purified RNA product using a NanoDrop spectrophotometer. Store RNA at –80 °C until ready to use.

3.3.2 RNA Transfection

1. Remove media from 100 mm plate of HeLa cells. Add 4 mL 1 \times PBS, wash, remove 1 \times PBS, and add 1 mL trypsin–EDTA. Allow plate to incubate in 37 °C incubator for 3 min. Wash off cells from plate with 9 mL of complete media.
2. Count cells and prepare 1.2×10^6 cells/transfection. Pellet cells at 150 $\times g$ for 4 min. Wash pellet with 1 \times PBS and pellet again. Suspend cells in ($n \times 400 \mu\text{L}$) 1 \times PBS. N = number of transfections needed.
3. Prepare 1.7 mL microcentrifuge tube with 5 μg of RNA transcript and place on ice. Do not add HeLa cells to RNA at this point.
4. Set Bio-Rad Gene Pulser Generator Model 1652076 (electroporator) to 0.13 kilovolts (kV) with (Bio-Rad Capacitance Extender Model 1652087) capacitance at 500 micro Farads (μFD). Remove cuvettes from individual wrapping and remove caps.
5. Add 400 μL of HeLa cell suspension to 1.7 mL tube of RNA. Quickly add the mixture to the cuvette and place cuvette into chamber and zap cells. Using media from 15 mL conical (pre-warmed from water bath) add 600 μL of media to zapped cells still in the cuvette. Gently pipet up and down multiple times to mix cells and media and to break up any cell clumps that may have formed.

Table 9
In-vitro T7 transcription reaction

Reagent	Volume (μL)	Reaction concentration
1 M HEPES 7.5	7	350 mM
320 mM Mg Acetate	2	32 mM
400 mM DTT	2	40 mM
20 mM Spermidine	2	2 mM
160 mM NTPs	3.5	28 mM
Linearized cDNA	2.5 (max volume)	0.025 $\mu\text{g}/\mu\text{L}$ (0.5 μg)
0.5 mg/mL T7 RNAP	1	0.025 $\mu\text{g}/\mu\text{L}$ (0.5 μg)
H ₂ O	2.5—DNA Volume	
<i>Reaction volume</i>	20	

3.3.3 Luciferase Assay

1. Prepare 15 mL conical tubes with 5.6 mL of complete media. Set them aside in 37 °C water bath until needed.
2. Proceed with RNA transfection as described previously (*see* RNA transfection).
3. Add 600 μL of media to zapped cells still in the cuvette. Gently pipet up and down multiple times to mix cells and media and to break up any cell clumps that may have formed.
4. Add cell and media mixture back to 15 mL conical tube. Close the tube and gently invert back and forth to mix. Aliquot 500 μL into a 1.7 mL tube and place the 15 mL conical in the 37 °C incubator until ready to take next timepoint.
5. Spin the one 1.7 mL tube at 2500 $\times g$ for 2 min for 0 h time point.
6. Remove media and wash pellet with 1 \times PBS. Spin again. Remove 1 \times PBS and add 100 μL of 1 \times CCLR (diluted to 1 \times in ddH₂O). Vortex tube for 10 s and place on ice.
7. Repeat this for every time point taken and leave all cells on ice until the next day. Make that each time an 500 μL aliquot is removed from the 15 mL conical tube that the tube is inverted multiple times to ensure an even distribution of cells.

8. Next day, vortex cells again for 10 s and spin down at max speed for 5 min to pellet any cellular debris.
9. Transfer 10 μL of supernatant to 12 \times 75 mm glass borosilicate assay tube. Let sit for 10–15 min then add 10 μL of luciferase substrate. Immediately place into luminometer and read light output.

3.3.4 Infectious Center Assay (See Quantitating virus and viral genomes components)

The infectious center assay is used to determine the fraction of cells within a culture that are infected with virus after RNA transfection. In this case, the infected cells are suspended, counted, and plated onto monolayers of susceptible cells, which are then overlaid with agar. The number of plaques provides a measure of the number of virus infected cells in the original culture, thus how infectious the *in vitro* transcribed RNA is.

1. One day before seed 6-well plates with 6×10^5 HeLa S3 cells per well and cover with 3 mL complete media.
2. Next day, follow procedure for *in vitro* RNA transfection.
3. Prepare the 6-well plate by removing media from each well. Wash with 1 \times PBS, remove PBS and add another 500 μL of PBS to each well and set to the side.
4. Serially dilute, tenfold, transfected cells in complete media. Place 100 μL of virus mixture on cells in 6-well plates.
5. Allow cells to adhere to the plate for 2 h at 37 $^\circ\text{C}$.
6. During incubation, prepare 2 \times complete media. Combine two packages of powdered media and 4.8 g of sodium bicarbonate into 1 L of ddH₂O. Adjust pH to 7.2 with concentrate HCl.
7. Inside a serological hood, filter media with 0.22 μm bottle top filter into autoclave 1 L pyrex media bottle. Next, prepare 2 % agarose solution using low melt agarose and ddH₂O. Make a 10 mL, 2 % solution for every 6-well plate prepared. Using a 500 mL Erlenmeyer flask, heat agarose mixture in microwave and be careful that solution doesn't boil over the top of the flask. Once all of the agarose is in solution, place in 37 $^\circ\text{C}$ water bath until cool enough to comfortably hold.
8. When cooled, add 20 % FBS and 200 U/mL of both penicillin and streptomycin then add the 2 \times media to final volume. Complete 2 \times media and 2 % agarose solution is at a 1:1 volume ratio in order to make final solution 1 \times complete DMEM and 1 % agarose. Completed agarose overlay can remain in the water bath until cells are ready.
9. After 2 h, cover cells with 3 mL of agarose overlay per well. Allow agarose to solidify at room temperature before incubating the plates at 37 $^\circ\text{C}$ for 2 days.

10. After 2 days of incubation, “pop” agarose plugs with metal spatula being careful not to scratch the cell monolayer. Once removed, wash cells with 1 mL 1× PBS. Remove PBS and add 500 µL of crystal violet stain. Let stand for 5 min, remove crystal violet and again wash with 1 mL 1× PBS. Count plaques to determine virus titer in pfu/mL.

3.3.5 Virus Isolation, Titer and One-Step Growth Curves

1. One day before, seed 100 mm plate with 3×10^6 HeLa S3 cells.
2. Next day, prepare 100 mm plate by removing media and washing once with 1× PBS. Add 9 mL of complete media to plate and put to the side.
3. Follow procedure for RNA transfection (*see* RNA transfection).
4. Add 1 mL of transfected cell mixture to HeLa cell monolayer and incubate at 37 °C until you observe cytopathic effects (CPE) under a light microscope. Complete CPE should be observed after 48 h (cells will be completely detached from the bottom of the plate and floating around in the media).
5. Upon CPE, harvest virus and cells into 15 mL conical and freeze on dry ice. Once frozen, thaw in 37 °C water bath then vortex for 30 s. Repeat this procedure two more times until you have performed “3 freeze-thaw cycles.”
6. Remove cell debris by centrifugation at top speed for 10 min. Pour off supernatant into a new 15 mL conical tube. Freeze on dry ice and store at -80 °C until ready to use (*see* **Note 5**).
7. This virus culture is labeled as passage 0 (P0).
8. To titer virus, prepare a 6-well plate, the day before, with 6×10^5 HeLa S3 cells per well and cover with 3 mL complete media.
9. Next day, serially dilute virus in PBS. Place 100 µL of virus mixture on cells in 6-well plates. Allow the virus to adsorb to the cells for 30 min.
10. After 30 min, remove virus and wash cells with 1 mL 1× PBS.
11. Remove PBS and replace with complete media containing 1 % agarose (*see* Infectious center assay for *how to make overlay*).
12. Allow agarose to solidify at room temperature before incubating the plates at 37 °C for 2 days.
13. After 2 days, “pop” agarose plugs with metal spatula being careful not to scratch the cell monolayer. Once removed, wash cells with 1 mL 1× PBS. Remove PBS and add 500 µL of crystal violet stain. Let stand for 5 min, remove crystal violet and again wash with 1 mL 1× PBS. Count plaques to determine virus titer in pfu/mL.
14. Also extract RNA using QIAamp Viral RNA mini kit to quantify genome copies/mL by RTqPCR (*see* Fecundity assay on *how to prepare the standard curve of RTqPCR*).

15. To make additional viral passages: One day before, seed 100 mm plate with 3×10^6 HeLa S3 cells.
16. Next day, prepare 100 mm plate by removing media and washing once with $1 \times$ PBS. Remove PBS and add 2 mL of fresh $1 \times$ PBS.
17. Inoculate cells with virus at MOI 0.01. Allow virus to adsorb to cells for 30 min, wash with $1 \times$ PBS and add 10 mL of complete to plate. Incubate at 37°C until CPE is observed (with WT PV CPE should be observed after 24 h, time to CPE mutants will vary).
18. Titer by plaque assay and quantitate genome copies/mL.
19. Keep passaging virus in this manor until you have reached the desired passage number. Sequence the 3Dpol gene to check for stability of the engineered mutation (*see Note 6*).
20. To analyze one-step virus growth, infected cells with virus at a multiplicity of infection (MOI) 10. Allow virus to adsorb to cells for 30 min and wash with $1 \times$ PBS then add 1 mL of complete media to cells.
21. Incubated at 37°C and harvest cells and media using a sterile disposable spatula (to scrape cells off the bottom of the well) at various time points post-infection. Virus and cell mixture is put into autoclaved 1.7 mL microcentrifuge tubes and immediately frozen on dry ice.
22. Harvest virus by three repeated freeze–thaw cycles and quantitate virus titers as described above.
23. Also extract RNA using Qiagen RNeasy Plus Mini Kit to determine genomes/mL by RT-qPCR.

3.3.6 Fecundity Assay

1. Using P0 virus, extract viral RNA using the QIAamp Viral RNA mini kit, following manufacturer's directions.
2. Determine viral genome copies by performing RT-qPCR on extracted virus sample.
3. Create a standard curve using in vitro transcribed RNA. Dilute RNA to $4\text{ ng}/\mu\text{L}$, which is approximately 1×10^9 genome copies/ μL . For a more accurate determination of genome copies/ μL , use digital PCR.
4. One day before seed 6-well plates with 6×10^5 HeLa S3 cells per well and cover with 3 mL complete media.
5. Infect HeLa cells with P0 virus that corresponds to a total of 3×10^2 , 3×10^3 , 3×10^4 , and 3×10^5 viral RNA genomes.
6. Incubate virus and cells at 37°C for 30 min. Remove virus and wash cells with 1 mL $1 \times$ PBS. Remove PBS and add 1 mL complete media to wells and allow viral replication to proceed for 8 h.

7. After 8 h, purify total RNA from infected cells with Qiagen RNeasy Plus Mini Kit.
8. Harvested virus is at passage 1 (P1). Harvest virus by three repeated freeze–thaw cycles. Perform RT-qPCR purified RNA to calculate the amount of virus required to infect next HeLa cells with a total of 3×10^2 , 3×10^3 , 3×10^4 , and 3×10^5 viral RNA genomes.

3.4 In Vivo Biological Analysis of Polymerase Mutants

3.4.1 Lethal Dose Fifty (LD_{50})/Paralysis Dose Fifty (PD_{50}) Analysis Components

1. House 4–6 week old outbred (ICR) mice transgenic for the PV receptor (cPVR) in standard ventilated caging for all experiments.
2. Passage 4 (P4) viral stocks are used for animal inoculations (*see* Virus isolation, titer and one-step growth curves).
3. Generate all virus stocks in serum-free media, harvested, titered, and genomes obtained.
4. Inoculate mice via intraperitoneal route (i.p.).
5. PD_{50} s are performed by infecting five mice per viral dose (1×10^7 , 1×10^8 , and 1×10^9 pfu) in order to calculate a PD_{50} .
6. Infect mice with virus by i.p. injection in 3 mL of serum-free media.
7. Observe mice for 14 days for signs of disease (ruffled fur and general malaise) and euthanize upon mice showing dual limb paralysis or paralysis such that their ability to obtain food and water is compromised.
8. After 14 days, determine PD_{50} values by the Reed and Muench method.

3.4.2 Protection

1. Mice previously infected with mutants, or surviving mice (from PD_{50} experiment) were challenged 1 month after initial infection using the same methods with $5 \times PD_{50}$ of WT poliovirus by i.p. injection and observed for 14 days as before.

4 Notes

1. The AAA codon encoding the lysine was changed to CGT codon encoding arginine. This genetic reversion requires two transversion mutations, which is a very inefficient event thus providing some barrier to reversion.
2. When using new primers, test the efficiency of the reaction by titrating in $MgSO_4$. Set up 3–100 μ L reactions and add 0, 1, and 2 μ L of the 100 mM $MgSO_4$. Run 10 μ L of completed product on agarose gel to check efficiency of reaction. Combine the successful reaction tubes and precipitate out DNA for gel purification.

3. Combine water, cDNA and buffer in 1.7 mL tube and before adding enzyme to your reaction, remove 5 μ L from the tube and set aside. This will be your “uncut” sample. Add the enzyme and allow reaction to proceed at proper temperature. After incubation remove another 5 μ L from the tube. This will be your “cut” sample.
4. The number of colonies after successful ligation and transformation of cloned pMoVRA and pRLucRA yield different results. When plating 100 % of your transformed cells, the pMoVRA clone should yield roughly 50 colonies on a plate, whereas the pRLucRA clone will yield at most 100 colonies.
5. Multiple freeze thaws of virus stock will overtime lower the titer of the virus. To avoid this, make small aliquots of virus and store at -80°C . Never use a stock tube that has been thawed more than three times after a titer or genome copy has been determined.
6. There are two reasons why it is important to passage virus at a low MOI. First is to check the stability of the engineered mutation by sequencing the mutated region; second is to generate a quasispecies.

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