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Poliovirus: Generation and Characterization of Mutants

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Abstract

Poliovirus (PV) is the prototypical picornavirus. It is a non-enveloped RNA virus with a small (~7.5 kb) genome of positive polarity. cDNA clones of several strains are available, and infectious virus can be produced by the transfection of *in vitro* transcribed viral genomes into an appropriate host cell. The ease of genetic studies in poliovirus is a primary reason that it has long served as a model to study RNA virus biology, pathogenesis, and evolution. Protocols for the generation and characterization of PV mutants are presented. A separate unit concerning the production, propagation, quantification, and purification of PV will also be presented.

Keywords

poliovirus; picornavirus; viral genetics; one-step growth curve; viral translation; viral replication

INTRODUCTION

Poliovirus (PV) is the causative agent of poliomyelitis and the founding member of the *Picornaviridae* family of small RNA viruses. The picornaviruses are non-enveloped viruses with single stranded RNA genomes of positive polarity. The PV genome is approximately 7400 nucleotides long and includes a 5′UTR followed by a single open reading frame and a polyadenylated 3′UTR. The open reading frame codes for the structural proteins that form the viral capsid as well as the nonstructural proteins, all of which are necessary for PV replication.

A large amount of research has examined the molecular biology of PV. A number of infectious cDNA clones, as well as *in vivo* and *in vitro* assays to study PV are available. PV has proven to be a useful model system and has helped shed light on many mechanisms of the positive-sense RNA virus life cycle.

The following protocols describe how to generate mutant viruses (or replicons) and the many assays one can use to compare them to wild type PV or other mutants. We start with how to examine the replication kinetics of PV in the presence and absence of drugs. Then we discuss methods to assay the translation and replication kinetics of viruses by uncoupling these processes, which are normally tightly linked. Assays for both *in vivo* and *in vitro* analysis are described. Next we move to genome replication, specifically, the synthesis

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positive- and negative-strand RNA from a single infectious cycle. Lastly, we present on two different methods for the analysis of viral protein synthesis.

CAUTION: Poliovirus (PV) is a Biosafety Level 2 (BSL-2) pathogen. Follow all appropriate guidelines and regulations for the use and handling of pathogenic microorganisms. See UNIT 1A.1 and other pertinent resources (APPENDIX 1B) for more information.

CAUTION: HeLa and HeLa S3 cells contain human papilloma virus (HPV-18), which is a Biosafety Level 2 (BSL-2) pathogen. Follow all appropriate guidelines and regulations for the use and handling of pathogenic microorganisms. See UNIT 1A.1 and other pertinent resources (APPENDIX 1B) for more information.

CAUTION: Take precautions to avoid contamination when working with radioactive materials. Carry out the experiment and dispose of wastes in designated areas, following guidelines provided by your institution's Radiation Safety Officer (also see *UNIT 7.1* and *APPENDIX 1D*) for more information.

Basic Protocol 1: GENERATION OF MUTANT cDNA PLASMIDS BY OVERLAP PCR AND RESTRICTION CLONING

Because PV is a (+) sense RNA virus, its RNA genome alone (Figure 1A) is sufficient to produce infectious virus, provided it can enter a host cell. Entrance (transfection) is achieved by electroporation. (See Unit 15H.1, Basic Protocol 1.) Similarly, transfection of a replicon, such as PLuc (Figure 1B), which contains the nonstructural viral genes as well as a luciferase reporter gene, can allow straightforward characterization of viral translation and replication kinetics (see Basic Protocol 4).

A basic PV (or PLuc) cDNA plasmid must contain a PV (or PLuc) genome with a T7 promoter upstream and a poly(A) tail downstream. For the poly (A) tail, a minimum of 8 'A's are essential for viral replication, and a longer tail will improve efficiency (Polacek & Andino, unpublished data). This protocol is based on methods used primarily for the plasmids prib(+)XpA and prib(+)RLuc (Figure 1C and D) (Herold and Andino, 2000). These plasmids contain the PV or PLuc genome (Type 1 Mahoney strain) in a pBR322 backbone. In addition to the T7 promoter and a long (75 nt) poly(A) tail, they also contain a hammerhead ribozyme sequence between the promoter start site and the genome. The ribozyme ensures an accurate 5′ end, which improves the efficiency of replication (Herold and Andino, 2000), but is not essential.

In the era of synthetic biology, it seems old-fashioned to describe a mutagenesis protocol based on restriction cloning. Synthetic biology has been used to great effect, for example to generate large-scale codon-shuffling mutants of PV (Mueller et al., 2006). Nonetheless, sometimes time, cost, or other considerations necessitate the use of simpler techniques. We have found this method to be more consistently reliable than QuikChange and other comparable methods.

This method is based on a two-step overlap PCR protocol, followed by conventional restriction cloning (Figure 2). It has been used successfully to introduce mutations spanning

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as many as 36 nucleotides at once. When designing mutagenesis primers, be sure to include "anchors" of at least 12 nucleotides of sequence identical to the template on either side of the mutagenesis target region. Mutagenesis primers may be exact reverse complements of each other, or they may be slightly offset, each having a longer 3′ anchor, as long as the overlap is at least 18 nucleotides. These criteria can result in long primers; up to 60 nucleotides is fine. Long primers tend to have high T_m values, so the PCR annealing temperature should be chosen based on the T_m values of the shorter PCR primers. At this lower temperature, both mutagenesis and PCR primers should anneal well.

Any PCR primers can be used as long as the intended restriction sites fall between the mutagenesis target region and the PCR primer binding sites. Given the scarcity of (unique) restriction sites within the PV genome, PCR products are often several kb in size. If possible, choose restriction enzymes that are compatible for a double digestion to save time, but if this is not possible sequential digestion is fine.

If introducing a mutation outside the P1 region of the genome into both PV and PLuc, it is often possible to use the same insert (steps 1–12) and simply perform simultaneous vector digests and ligations (steps 13–32). In this case, be sure to choose restriction sites that are unique in both plasmids.

Materials

10 ng/μL cDNA plasmid template

10 μM For PCR primer

10 μM Rev PCR primer

10 μM For mutagenesis primer

10 μM Rev mutagenesis primer

Herculase II Fusion DNA Polymerase

5× Herculase II reaction buffer (provided with Herculase II Fusion DNA Polymerase)

dNTP mix (10 mM each dNTP)

Nuclease-free water

1% agarose TAE gels and running buffer (Voytas, 2001)

6× DNA Loading Dye

DNA Ladder (e.g. GeneRuler 1 kb Plus, Fermentas)

Gel and PCR Cleanup Kit (e.g. Nucleospin Gel and PCR Clean-up, Machery-Nagel)

10 μg cDNA plasmid vector

Restriction enzymes

Buffer(s) for restriction enzymes (provided with enzyme(s))

BSA (if needed; provided with enzyme(s))

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5 U/μL Antarctic Phosphatase (NEB)

10× Antarctic Phosphatase Reaction Buffer (provided with 5U/μL Antarctic Phosphatase)

Quick Ligation Kit (NEB; includes Quick T4 DNA Ligase and 2× Quick Ligation Reaction Buffer)

SURE Electroporation-competent Cells (Agilent)

Sterile water

LB medium

LB-Amp agar plates (50–100 μg/mL)

LB-Amp medium (50–100 μg/mL)

Mini-prep Kit (e.g. NucleoSpin Plasmid, Machery-Nagel)

1× TE (pH 8.0)

Ice

Electroporator and 1 mm cuvettes

Perform first round overlap PCR

1. Prepare 2 separate PCR reactions, one with each of these primer pairs: For PCR primer & Rev mutagenesis primer, For mutagenesis primer & Rev PCR primer. Mix (per reaction):

   10.0 μL 5× Herculase II reaction buffer

   1.25 μL dNTP mix

   3.0 μL 10 ng/μL cDNA plasmid template

   1.25 μL 10 μM For primer

   1.25 μL 10 μM Rev primer

   1.0 μL Herculase II Fusion DNA Polymerase*

   32.25 μL nuclease-free water*

2. Mix well and perform PCR:

First step:	2:00	95°C	(initial denaturation)
30 cycles:	0:20	95°C	(denaturation)
	0:20	PCR primer T_m - 5°C	(annealing)
	0:30	72°C	(extension 30 s per kb)
Final step:	3:00	72°C	(final extension)

*If first round PCR product is less than 1 kb in length, adjust to 0.5 μL polymerase and 32.75 μL water.

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3. Run 5 μL of each PCR reaction with 1 μL 6× DNA loading dye on a 1% agarose TAE gel (Voytas, 2001) and compare to DNA ladder to confirm size.

4. Perform PCR cleanup using a spin column kit such as the Nucleospin Gel and PCR Clean-up Kit (Machery-Nagel). Elute in 50 μL elution buffer.

5. Check DNA concentration on NanoDrop spectrophotometer. Adjust to 0.1 pmol/μL with elution buffer.

   The NanoDrop gives concentrations in ng/μL. To convert to pmol/μL, first calculate the molecular weight of your PCR product in ng/pmol. [M.W. (ng/pmol) = length (bp) × 0.6074 + 0.1579.] Divide concentration (ng/μL) by molecular weight (ng/pmol) to get pmol/μL. Store DNA at −20°C.

Perform second round overlap PCR

6. Prepare a single PCR reaction combining 0.3 pmol of each of the first round products. Mix (per reaction):

   10.0 μL 5× Herculase II reaction buffer

   1.25 μL dNTP mix

   3.0 μL 0.1 pmol/μL For PCR & Rev mutagenesis product

   3.0 μL 0.1 pmol/μL For mutagenesis & Rev PCR product

   1.0 μL Herculase II Fusion DNA Polymerase*

   29.25 μL nuclease-free water*

7. Mix well and perform PCR:

First step:	2:00	95°C	(initial denaturation)
10 cycles:	0:20	95°C	(denaturation)
	0:20	PCR primer T_m - 5°C	(annealing)
	0:30	72°C	(extension 30 s per kb) *
Final step:	3:00	72°C	(final extension)

*Calculate extension time based on the distance between the mutagenesis primers and the further of the two PCR primers; therefore it should be the same as for the first round of PCR.

8. Add 1.25 μL 10 μM For PCR primer and 1.25 μL 10 μM Rev PCR primer to the reaction.

*If second round PCR product is less than 1 kb in length, adjust to 0.5 μL polymerase and 29.75 μL water.

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9 Mix well and perform PCR:

First step:	2:00	95°C	(initial denaturation)
20 cycles:	0:20	95°C	(denaturation)
	0:20	PCR primer T_m - 5°C	(annealing)
	0:30	72°C	(extension 30 s per kb) *
Final step:	3:00	72°C	(final extension)

*Calculate extension time based on the distance between the PCR primers; therefore it may be longer than for the previous steps.

10 Run 5 μL of PCR reaction with 1 μL 6× DNA loading dye on a 1% agarose TAE gel (Voytas, 2001) and compare to DNA ladder to confirm size.

11 Perform PCR cleanup using a spin column kit such as the Nucleospin Gel and PCR Clean-up Kit (Machery-Nagel). Elute in 50 μL elution buffer.

Store DNA at −20°C.

Digest and gel purify inserts (PCR products) and vectors

NOTE: Final digest volumes of 65 μL are based on the volume that can easily be combined with 6× loading dye and loaded into a single well for gel purification. Adjust volumes up or down based on your well size. If performing a double digest, check the concentration of both enzymes and add an equal number of units of each. Restriction digests should not contain more than 5% glycerol. Since restriction enzymes are typically supplied in 50% glycerol, enzymes should not make up more than 10% of the final volume of the digest reaction. For example, to perform a double digest in 65 μL with *Bmg*BI supplied at 10 U/μL and *Bgl*II supplied at 50 U/μL, add up to 54 U of each: 5.42 μL *Bmg*BI + 1.08 μL *Bgl*II = 6.50 μL total enzyme (10% of final volume).

12 Prepare digest of insert. Combine second round PCR product, buffer, BSA if needed, and enzyme(s) to a final volume of 65 μL. Incubate 1 h at 37°C (or other appropriate temperature for the restriction enzyme in question).

13 Prepare digest of vector. Combine at least 10 μg cDNA plasmid vector, buffer, BSA if needed, and enzyme(s) to a final volume of 57.5 μL. Incubate 1 h at 37°C (or other appropriate temperature for the restriction enzyme in question).

14 If performing a sequential digest, cleanup both digest reactions with a spin-column kit such as the Nucleospin Gel and PCR Clean-up Kit (Machery-Nagel) and repeat steps 12 and 13 with the second enzyme. If performing a double digest, proceed with step 15.

15 To the vector digest, add 6.5 μL 10× Antarctic Phosphatase Reaction Buffer and 1.0 μL 5U/μL Antarctic Phosphatase. Incubate 15 min at 37°C.

16 Combine each digest reaction with 13 μL 6× DNA Loading Dye. Perform electrophoresis in a 1% agarose TAE gel (Voytas, 2001) to separate fragments.

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17 Compare bands to DNA ladder to confirm size. Using a clean razor blade, cut out digested vector and insert bands and place gel slices in separate 1.5 mL tubes.

18 Perform gel cleanup using a spin column kit such as the Nucleospin Gel and PCR Clean-up Kit (Machery-Nagel). Elute in 50 μL elution buffer.

19 Check DNA concentration on NanoDrop spectrophotometer.

Store DNA at −20°C.

Ligate insert and vector and transform ligation into SURE cells

20 Prepare two 0.5 mL tubes and add 50 ng digested and dephosphorylated vector to each. To one tube, add a 3-fold molar excess of digested insert (this is the ligation tube). To the other tube, add a similar volume of elution buffer (this is the vector control tube). Adjust the final volume of each tube to 10 μL with nuclease-free water.

21 Add 10 μL 2× Quick Ligation Reaction Buffer and 1 μL Quick T4 DNA Ligase to each tube. Incubate 15 min at room temperature.

22 Cleanup ligation and control reactions using a spin column kit such as the Nucleospin Gel and PCR Clean-up Kit (Machery-Nagel). Elute in 20 μL water. Chill on ice.

23 Chill a 1 mm electroporation cuvette on ice. Thaw SURE cells on ice.

24 Add 20 μL SURE cells to ligation and control reactions and mix gently with pipette tip. Transfer to 1 mm cuvettes.

After first thaw, refreeze extra SURE cells in single-use aliquots to avoid repeated freeze-thaw cycles.

25 Electroporate (voltage = 1.8 kV, resistance = 129 Ω) cells and recover in 960 mL LB-Amp. Transfer to 1.5 mL tube.

26 Incubate 1 h at 37°C with shaking.

27 Plate 100 μL of each reaction on an LB-Amp agar plate. Centrifuge 5 min at 2,000× *g* to pellet remaining cells. Remove ~800 μL of supernatant, resuspend cells in remaining ~100 μL, and plate on LB-Amp agar plate.

28 Incubate plates overnight at 37°C.

Prep and sequence colonies

The number of colonies to pick and sequence can be estimated by examining the vector control plate. For example, if the vector control plate has 20 colonies and the ligation plate has 100 colonies, you can estimate that approximately 20% of the colonies on the ligation plate will be due to vector auto-ligation. The remaining 80% will contain inserts, but not always perfect inserts; there may be insertions, deletions, or point mutations from the PCR steps. Typically 5–10 clones is enough to ensure one clean insertion; occasionally 20 or more may be needed.

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29 Use sterile pipet tips to pick 5–10 colonies and inoculate 3 mL LB-Amp cultures. Incubate overnight at 37°C with shaking.

30 Perform plasmid mini-prep according to kit instructions. Quantitate the DNA on a NanoDrop Spectrophotometer.

31 Adjust DNA concentration according to the sequencing facility parameters and sequence entire insert, including restriction sites.

Be sure to reserve enough of each prep to allow transformation of the clone containing the correct mutation (and no others) back into SURE cells.

32 Analyze sequences and retransform and prep good clone (see Unit 15H.1 Support Protocol 1).

Basic Protocol 2: ONE-STEP GROWTH CURVES

A one-step growth curve is one of the most common ways of characterizing a mutant virus. Although common, it is not a terribly sensitive assay. Viruses with minor defects in one or more viral processes may not exhibit gross replication defects observable by this method. Using 24-well plates, it is possible to assay up to 8 viruses in triplicate (or fewer viruses with more replicates). We typically take eight time points – 0, 2, 4, 6, 8, 10, 12, and 24 hours post-infection.

Materials

Virus stocks of known titer to be assayed

HeLa or HeLa S3 cells (Unit 15H.1, Support Protocol 2)

10% NCS DMEM/F-12

Serum-free DMEM/F-12

24-well plates

37°C and 5% CO_2 humidified incubator

Prepare cells and virus

1 Seed HeLa or HeLa S3 cells in 24-well plates at 2.5×10^5 cells in 0.5 mL of 10% NCS DMEM/F-12 per well and incubate 24 h at 37°C in 5% CO_2, at which time they should be confluent. Prepare 1 plate for each time point to be taken.

The pros and cons of HeLa vs. HeLa S3 cells are discussed in Unit 15H.1, Support Protocol 2.

2 On the day of the assay, thaw virus stocks and dilute to 5×10^7 PFU/mL in cold serum-free DMEM/F-12. You will need 100 μL inoculum per replicate (e.g. triplicate samples × 8 time points = 24 replicates) plus 5–10% extra to account for pipetting error. 3 mL will allow for 27–28 replicates.

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This concentration gives a multiplicity of infection (MOI) of ~10. A confluent well has ~ 5×10^5 cells. For an MOI of ~10, you need ~5×10^6 PFU/0.1 mL inoculum = ~ 5×10^7 PFU/mL. A high MOI is necessary to ensure one-step growth kinetics, but if stock titers are too low it can be reduced to ~5.

Infect cells and take time points

3. Aspirate medium and wash cells in 0.5 mL/well cold PBS.
4. Aspirate PBS in all wells to be infected with first virus. Add 100 μL inoculum to each well.

   Each plate represents a time point and should be identical at this point. All viruses infect 3 or more wells on each plate.

5. Repeat step 4 for each subsequent virus.
6. Incubate 30 min at 4°C to adsorb virus.

   At 4°C, PV will bind to the poliovirus receptor (PVR) but further steps of the infection will not proceed. This ensures that the infections are launched synchronously.

7. Wash cells twice with 0.5 mL/well cold PBS.
8. Cover cells in 0.5 mL/well serum-free DMEM/F-12. Place one plate (0 h time point) at −20°C and the rest at 37°C in 5% CO_2.
9. At each time point, transfer one plate from 37°C to −20°C.

Harvest samples and titer

10. When all time points have been frozen, thaw the plates at room temperature. Then, return to −20°C to freeze again.
11. Thaw the plates at room temperature again and transfer each sample to a 1.5 mL tube. Freeze samples at −20°C.
12. Titer samples by plaque assay or $TCID_{50}$ (see Unit 15H.1, Basic Protocol 2 or Alternate Protocol 1). Immediately before titering thaw samples to room temperature and centrifuge 5 min at 12,000×*g* to pellet cellular debris.
13. Plot time post-infection vs. log viral titer.

    We perform a t-test to determine whether any observed differences are significant. Differences of less than half a log are not typically significant due to the relatively high margin of error when measuring viral titer.

Basic Protocol 3: DRUG RESISTANCE CURVES

Drug resistance curves are another common way of characterizing a mutant virus. It is especially useful when characterizing a drug resistance phenotype or the response of a

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mutant virus to a whole class of drugs, such as mutagens. Using 24-well plates, it is convenient to assay a no drug control plus up to 7 drug concentrations in triplicate (or fewer concentrations with more replicates; we commonly assay no drug plus 5 concentrations in quadruplicate).

Materials

Virus stocks of known titer to be assayed

Drug to be tested

HeLa or HeLa S3 cells (Unit 15H.1, Support Protocol 2)

10% NCS DMEM/F-12

Serum-free DMEM/F-12

24-well plates

37ºC and 5% CO_2 humidified incubator

Prepare cells, drug, and virus

1 Seed HeLa or HeLa S3 cells in 24-well plates at 2.5×10^5 cells in 0.5 mL of 10% NCS DMEM/F-12 per well and incubate 24 h at 37ºC in 5% CO_2, at which time they should be confluent. Prepare 1 plate for each virus to be assayed.

2 Prepare concentrated drug stock in sterile water, DMSO, or other convenient solvent. This stock should be at least 100× more concentrated than the highest drug concentration you intend to test. For example, if you intend to test the effect of 1000 μM drug on the virus, your stock should be at least 100 mM.

3 Prepare dilutions of the drug in serum-free medium at the concentrations of interest. Add the same volume of drug stock + solvent to each dilution. You will need 1 mL of dilution for each replicate (e.g. quadruplicate samples × 4 viruses = 16 replicates) plus 5–10% extra to account for pipetting error. 10 mL will allow for 18–19 replicates. These dilutions can normally be stored at 4ºC overnight, but it is wise to confirm the stability of the drug in question.

Determining which drug concentrations to test requires careful consideration. First determine a maximum concentration. You may wish to consult the literature or do some preliminary experiments to determine whether high concentrations of the drug are unduly toxic to HeLa or HeLa S3 cells. Having chosen a maximum concentration (e.g. 1000 μM), you should then determine whether you want test an even range of concentrations (e.g. 0, 200, 400, 600, 800, and 1000 μM) or a skewed range that tests more low concentrations (e.g. 0, 50, 100, 200, 500, and 1000 μM). If the virus is particularly sensitive to the drug, low concentrations may have significant effects while the difference between the effects of two high concentrations may be small.

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4 On the day of the assay, pretreat the cells with the drug. Aspirate the old medium and replace with 0.5 mL/well of the appropriate dilution of drug in serum-free medium. The time of pretreatment may vary somewhat with the drug, but 4 h is usually sufficient. Incubate at 37ºC and 5% CO_2.

Each plate represents a virus and therefore should have a complete set of replicate drug dilutions.

5 Thaw virus stocks and dilute to 5×10^7 PFU/mL in cold serum-free DMEM/F-12. You will need 100 μL inoculum per replicate (e.g. quadruplicate samples × 6 drug concentrations = 24 replicates) plus 5–10% extra to account for pipetting error. 3 mL will allow for 27–28 replicates.

This concentration gives a multiplicity of infection (MOI) of ~10. A confluent well has ~ 5×10^5 cells. For an MOI of ~10, you need ~5×10^6 PFU/0.1 mL inoculum = ~ 5×10^7 PFU/mL. If you wish to test the effect of the drug on more than one round of replication, it is necessary to use a low MOI instead (<1).

Infect cells

6 Aspirate medium from one plate and add 100 μL of the first viral inoculum to each well.

7 Repeat step 6 for each subsequent plate and virus.

8 Incubate 30 min at 4ºC to adsorb virus.

At 4ºC, PV will bind to the poliovirus receptor (PVR) but further steps of the infection will not proceed. This ensures that the infections are launched synchronously.

9 Aspirate inoculum and replace with 0.5 mL/well of the appropriate dilution of drug in serum-free medium.

10 Incubate at least 8 h at 37ºC and 5% CO_2.

11 Confirm the presence of CPE in the no drug wells using a tissue-culture microscope. When CPE is present, freeze plates at −20ºC.

Harvest samples and titer

12 Thaw the plates at room temperature. Then, return to −20ºC to freeze again.

13 Thaw the plates at room temperature again and transfer each sample to a 1.5 mL tube. Freeze samples at −20ºC.

14 Titer samples by plaque assay or $TCID_{50}$ (see Unit 15H.1, Basic Protocol 2 or Alternate Protocol 1). Immediately before titering thaw samples to room temperature and centrifuge 5 min at 12,000×*g* to pellet cellular debris.

15 Plot drug concentration vs. log viral titer.

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We perform a t-test to determine whether an observed differences are significant. Differences of less than half a log are not typically significant due to the relatively high margin of error when measuring viral titer.

Basic Protocol 4: ASSAYING TRANSLATION AND REPLICATION WITH PLUC REPLICON

Creation of PV luciferase replicon (PLuc; Figure 1B) has made it possible to study the effect of lethal mutations, which are impossible to study in live virus, on replication and translation (Andino et al., 1993). Capsid proteins are not essential for viral replication (Hagino-Yamagishi and Nomoto, 1989; Kaplan and Racaniello, 1988), therefore, it was possible to replace the entire capsid region (P1; nucleotides 743-3385) with a luciferase reporter gene. Translation of this construct yields luciferase protein followed by all the nonstructural PV proteins (P2 and P3). Because the viral open reading frame is translated as one large polyprotein, the expression of the reporter gene is proportional to the amount of viral RNA present in the cell and therefore acts as a measure of viral RNA synthesis.

The prib(+)RLuc plasmid (Figure 1D) contains a luciferase reporter gene in place of the P1 capsid region of poliovirus type 1 cDNA. Immediately following the luciferase gene, an artificial $2A^{pro}$ cleavage site was engineered, allowing for the release of the active luciferase enzyme from the rest of the polyprotein. It is otherwise identical to the prib(+)XpA plasmid (see Unit 15H.1, Basic Protocol 1 and Support Protocol 1) (Herold and Andino, 2000).

Millimolar concentrations of guanidine hydrochloride (GuHCl) have been shown to reversibly inhibit poliovirus replication. The drug interacts with the nucleotide-binding region of poliovirus $2C^{ATPase}$(Crowther and Melnick, 1961; Pincus et al., 1986; Rightsel et al., 1961; Tolskaya et al., 1994). Viral translation, however, remains unchanged. The use of GuHCl makes it is possible to uncouple the viral replication and translation processes.

The PLuc replicon assay, performed in the presence and absence of GuHCl, can determine if mutant viruses are deficient in replication, translation or both processes. This is a powerful tool for the analysis of mutant viruses.

Materials

PLuc plasmid prib(+)RLuc*

10 U/μL *Mlu*I (New England BioLabs)

10× NEBuffer 3 (provided with *Mlu*I)

Nuclease-free water

1% agarose TAE gels and running buffer (Voytas, 2001)

6× DNA Loading Dye

DNA Ladder (e.g. GeneRuler 1 kb Plus, Fermentas)

Phenol:chloroform:IAA (25:24:1)

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Chloroform:IAA (24:1)

3 M NaOAc (pH 5.2)

Isopropanol

75% (v/v) EtOH

T7 RNA Polymerase**

5× Transcription Buffer**

40 U/µL RNaseOUT (Invitrogen)

NTP Mix (25 mM each NTP)

2U/µL DNase I (RNase-free; New England BioLabs)

3× LiCl-EDTA

0.5× TE

2× RNA Denaturing Loading Dye

HeLa or HeLa S3 Cells (Unit 15H.1, Support Protocol 2)

D-PBS (PBS w/o Ca^{2+} or Mg^{2+})

0.05% Trypsin-EDTA

10% NCS DMEM/F-12

2M GuHCl

6-well plates

5× Cell Culture Lysis Reagent (Promega)

Luciferase Assay System (Promega)

37ºC heat block or water bath

95ºC heat block

Ice

NanoDrop spectrophotometer

37ºC and 5% CO_2 humidified incubator

Electroporator and 4 mm cuvettes

Tissue culture microscope (inverted phase-contrast)

Linearize plasmid

PLuc plasmids typically contain several unique restriction sites immediately downstream of the encoded poly(A) tail. In the case of our most commonly used plasmid, prib(+)RLuc (Herold and Andino, 2000), the best site is *Mlu*I. A minimum of 10 µg of DNA is required. We typically digest 25 µg in a 500 µL volume. After phenol-chloroform extraction, this yields enough linearized plasmid to template 3–4 *in vitro* transcription reactions.

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**NOTE:* prib(+)RLuc plasmid propagation and maintenance is identical to that of prib(+)XpA; see Unit 15H.1, Support Protocol 1.

1. Combine 25 μg plasmid DNA, 50 μL 10× NEBuffer 3, 125 U *Mlu*I, and nuclease-free water in a 500 μL final volume and incubate 1 h at 37°C.

2. Remove 5 μL (1%) of digest reaction. Dilute 500 ng undigested plasmid DNA to 5 μL. Add 1 μL 6× DNA Loading Dye to each and analyze by electrophoresis in a 1% agarose TAE gel (Voytas, 2001).

   If digestion is complete (all digested DNA should be converted to a single linear fragment of ~10 kbp, distinguishable from the multiple circular DNA forms of the undigested plasmid), proceed to step 4.

3. If digestion is not complete, redigest with 125 U fresh *Mlu*I and repeat step 3.

4. Perform phenol:chloroform extraction:

   a. Add an equal volume (500 μL) of phenol:chloroform:IAA and mix. Centrifuge 10 min at 12,000× *g*. Transfer aqueous phase to a fresh tube.

   b. Add an equal volume (500 μL) of chloroform:IAA and mix. Centrifuge 10 min at 12,000× *g*. Transfer aqueous phase to a fresh tube.

   c. To precipitate the DNA, add 0.1 volume (50 μL) 3M NaOAc and 1 volume (500 μL) isopropanol. Incubate at least 20 min at −20°C. To pellet the DNA, centrifuge 20 min at 12,000× *g*, 4°C.

   d. Discard the supernatant and wash the pellet in 500 μL 75% EtOH. Centrifuge 5 min at 7,500× *g*.

   e. Discard the supernatant and remove the residual liquid with a pipette. Resuspend the DNA in 25 μL nuclease-free water. Quantitate the DNA on a NanoDrop Spectrophotometer and adjust the concentration to 500 ng/μL.

      Store linearized DNA at −20°C.

In vitro transcribe replicon RNA

Most PLuc plasmids contain an T7 promoter upstream of the viral genome and the ribozyme if applicable. A minimum of 1 μg linearized DNA is required. We typically use 5 μg template in a 100 μL volume. Yield is highly variable. After lithium chloride precipitation, there is usually enough for 1–4 transfections.

***NOTE:* We use an in-house preparation of T7 RNA Polymerase with 5× Transcription Buffer. The enzyme is also available commercially from a number a sources and is typically provided with a 10× Tris-based buffer.

5. Combine 5 μg linearized plasmid DNA, 20 μL 5× Transcription Buffer, 30 μL NTP Mix, 40 U RNaseOUT, 1 μL T7 RNA Polymerase, and nuclease-free water in a 100 μL final volume and incubate 4–16 h at 37°C.

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Longer incubation times increase yield at the expense of some level of RNA degradation. Overnight incubation produces RNA of sufficient quality to generate virus. If a precise quantification of transcripts is necessary, RNA of higher quality (but lower yield) is obtained from shorter incubations.

6 To digest the DNA template, add 5U DNase I and incubate 10 min at 37°C.

7 To selectively precipitate the RNA transcript (but not the free nucleotides), add 0.5 volumes (52.5 μL) 3× LiCl-EDTA and incubate at least 1 h at −20°C. To pellet the RNA, centrifuge 20 min at 12,000× *g*, 4°C.

8 Discard the supernatant, remove the residual liquid with a pipette, and wash the pellet in 500 μL 75% EtOH. Centrifuge 5 min at 7,500× *g*, 4°C.

9 Discard the supernatant and remove the residual liquid with a pipette. Resuspend the RNA in 25 μL 0.5× TE. Quantitate a 1:10 dilution of the RNA on a NanoDrop Spectrophotometer and adjust the concentration to 1 μg/μL.

Store in vitro transcribed RNA at −70°C.

10 Dilute 1 μg RNA to 3 μL and add 3 μL Gel Loading Buffer II. Incubate 5 min at 95°C, then 2 min on ice. Analyze by electrophoresis in a 1% agarose TAE gel (Voytas, 2001).

If RNA quality is good, this "semi-denaturing" gel analysis (denaturing buffer, non-denaturing gel) will show a clean single band. RNA degradation is seen as smearing below the main band. Smearing above the main band is usually due to incomplete linearization of plasmid template. Poor quality RNA will usually still generate virus upon transfection, but should not be used if precise quantification of transcripts is important (e.g. to calculate transfection efficiency or generate a qPCR standard curve).

Transfect replicon RNA

Many cells are lost during the washes. Typically a confluent T150 flask provides enough cells for 3–5 transfections. We use a BTX Electro Cell Manipulator 600 with the pulse settings described below. Settings may vary depending on the model of electroporator used.

11 Wash HeLa or HeLa S3 monolayer with D-PBS and trypsinize cells with 3 mL 0.05% Trypsin-EDTA.

12 Resuspend cells in 15 mL D-PBS. Centrifuge 5 min at 200× *g*. Aspirate or pour off supernatant.

13 Repeat step 2 twice more.

Three washes are generally sufficient to remove the majority of salt from the cells. More washes may be done to avoid the possibility of arcing upon electroporation. If arcing does occur, the viability of the

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cells will be greatly decreased. It is best to repeat the protocol with an increased number of washes if this occurs.

14 After the third spin, resuspend the cells in 2 mL D-PBS and count on a hemocytometer. Adjust the concentration to 7×10^6 cells/mL.

The cells will be very concentrated in 2mL of D-PBS. Dilute cells 1/100 prior to counting.

15 Combine 800 μL of cells and 20 μg of RNA in a chilled 4 mm electroporation cuvette and incubate 20 min on ice.

16 Electroporate (voltage = 300 V, capacitance = 1000 μF, resistance = 24 Ω) cells and recover in 15 mL 37ºC 10% NCS DMEM/F-12.

17 Equally divide recovered cells into two 15 mL conical tubes (approximately 7.5 mL/tube). Add 7.5 μL 2 M GuHCl to one of the identical tubes.

In the presence of GuHCl poliovirus will not replicate. These samples will determine the amount of translation occurring in the mutants. Samples lacking GuHCl will determine the amount of both replication and translation occurring in the mutants.

At this point, it is possible to leave the samples in 15 mL tubes and incubate at 37ºC in a tube rotator. This approach allows greater flexibility in determining the number and frequency of time point samples to collect. A more standard practice is to plate seven 1 mL aliquots and take hourly time points for seven hours. If you wish to leave them in 15 mL conicals, then simply remove a fixed volume (typically 500 μL) to a 1.5 mL tube at each time point and skip to step 20.

18 Plate 1 mL aliquots of recovered cells with and without GuHCl onto 6-well plates. Incubate at 37C and 5% CO_2. Collect time points every hour.

There should be a total of seven 6-well plates for up to three mutants. It is important to prepare one plate for each time point. Removing the time points from the incubator repeatedly prior to collection will bias your results.

Collect and analyze time point samples

19 Using a cell scraper, gently remove cells from the 6-well plate and transfer to a 1.5 mL tube.

20 To pellet the cells, centrifuge 1 min at 1000× *g*. Carefully remove supernatant and resuspend cells in 60 μL 1× Cell Culture Lysis Reagent. Incubate 2 min on ice.

Dilute 5× Cell Culture Lysis Buffer to 1× concentration prior to use.

21 To pellet cellular debris, centrifuge 1 min at 12,000× *g*. Transfer supernatant (~50 μL) to clean 1.5 mL tube.

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Store time point samples at −20°C until ready for luciferase analysis.

22 Analyze luciferase activity in time point samples using a kit such as the Luciferase Assay System (Promega).

Basic Protocol 5: *IN VITRO* TRANSLATION AND REPLICATION (IVTR) ASSAY WITH S10 HeLa EXTRACTS

The ability to form functional RNA replication complexes and produce infectious virus from HeLa cell extract has greatly advanced the field of PV research.

S10 extracts from HeLa S3 cells are essential for studying PV replication and translation *in vitro.* The extract provides a complete system where replication complex formation, translation, protein processing, RNA replication and virus assembly can occur (Barton et al., 1995). Using the cell-free system, radiolabeled PV intermediates are detected by autoradiography and defects at particular points in the virus life cycle can be easily determined.

Guanidine hydrochloride (GuHCl) has been shown to reversibly inhibit poliovirus replication when used at millimolar concentrations by interacting with the nucleotide-binding region of PV $2C^{ATPase}$ (Crowther and Melnick, 1961; Pincus et al., 1986; Rightsel et al., 1961; Tolskaya et al., 1994). Translation of the viral RNA, however, remains unaffected. The use of GuHCl in the cell free system makes it is possible to synchronize viral replication by allowing translation of the viral genome and formation of the pre-initiation RNA replication complexes. These complexes contain all the factors required for viral RNA replication. Upon removal GuHCl the complexes are capable of initiating replication synchronously.

Production of extract that is capable of supporting translation and replication can be challenging. It is extremely important to control the pH of buffers used during extract preparation. In situations where the viral RNA does not translate or replicate it is most likely due to the concentration of Mg^{2+} in the cells. Small deviations from optimal conditions will dramatically reduce the amount of protein synthesis and therefore viral replication.

Materials

10× NTP-Rx Buffer without UTP

100 mM GuHCl

S10 HeLa Extract (Support Protocol 1)

Initiation Factors (Support Protocol 2)

DEPC-treated water

In vitro transcribed viral and/or replicon RNA (Basic Protocol 4 and/or Unit 15H.1, Basic Protocol 1)**

Puromycin

30 μCi ^{32}P-UTP (PerkinElmer)

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TENSK Buffer

Phenol:chloroform:IAA (25:24:1)

70% (v/v) EtOH

99% (v/v) EtOH

3 M NaOAc (pH 5.5)

6× Gel Loading Buffer (RNase free)

0.8% agarose TBE gels and running buffer (Voytas, 2001)

5× Cell Culture Lysis Reagent (Promega)**

Luciferase Assay System (Promega)**

30ºC heat block or water bath

Set up translation

1 Prepare the following master mix for *in vitro* translation in a 1.5 mL tube:

5 μL 10× NTP-Rx buffer without UTP

1 μL 100 mM GuHCl

3 μL S10 HeLa extract (Support Protocol 1)

2 μL Initiation Factors (Support Protocol 2)

DEPC-treated water to 50 μL final volume

2 Add 2 μg of *in vitro* transcribed viral RNA.

Viral RNA can be synthesized by in vitro transcription described in Unit 15H.1 Basic Protocol 1. It is also possible to use PV replicon RNA (Basic Protocol 4) that contains the luciferase gene instead of the P1 capsid region. We generally adjust stock viral RNA to a concentration of 1 μg/μL and add 2 μL.

3 Incubate 4 h at 30°C.

**When using replicon RNA it is possible take 1 μL of the sample at 3 h 45 min to check for successful translation. Add to 50 μL Cell Culture Lysis Reagent (Promega) to the 1 μL sample. Use 10 μL of this mixture to measure luciferase activity with the Luciferase Assay System (Promega).

4 Centrifuge 15 min at 12,000× *g*, 4°C. Carefully remove supernatant without disturbing the pellet.

Removal of supernatant is essential because it contains GuHCl, which inhibits replication. The initial inclusion of GuHCl is necessary, however, to synchronize replication.

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Set up replication

5 Prepare the following master mix for *in vitro* replication in a 1.5 mL tube:

2 μL DEPC-treated water

2.5 μL 10× NTP-Rx buffer without UTP

2.5 μL Puromycin

3 μL ^{32}P-UTP (30 μCi)

15 μL S10 HeLa extract

When working with ^{32}P, tubes should be shielded with ¼–½ inch Plexiglas. Take care to minimize hand exposure to radioactivity. Be sure to dispose of all waste from this point forward according to both Biological and Radioactive safety guidelines.

6 Carefully add 50 μL master mix to the pellet.

Add the master mix slowly next to the pellet. If the pellet floats in the reaction mix this is acceptable. Do not pipet up and down; this will destroy the pellet and therefore replication.

7 Incubate 2 h at 30°C.

Prepare and visualize RNA

8 Stop reaction by adding 175 μL TENSK buffer to each 50 μL reaction. Pipette up and down to mix. Incubate 1 h at 42°C.

9 Add 1 volume (225 μL) of Phenol:Chloroform:IAA, vortex, and centrifuge 15 min at 12,000× *g*, 4°C.

10 Transfer aqueous (upper) phase to a 1.5 mL tube. Add 20 μL 3 M NaOAc (pH 5.5) and 500 μL 99% EtOH, then vortex.

11 Centrifuge 15 min at 12,000× *g*, 4°C. Remove and discard supernatant.

12 Wash pellet by adding 500 μL 70% EtOH and centrifuge 5 min at 12,000× *g*, 4°C.

13 Dry pellet by exposing it to air for 5 min, then resuspend in 50 μL DEPC-treated water.

Store samples at −70ºC.

14 Load 15 μL of RNA on a native 0.8% agarose TBE gel. Run gel for 4 h at 55 V (Voytas, 2001).

For sharper bands it is possible to run the RNA on a 0.8% agarose TBE gel overnight at 20 V.

15 Dry gel under vacuum for 60–90 min at 65°C.

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16. Visualize radiolabeled RNA products by autoradiography and quantify bands using a quantitative imaging device (e.g. Typhoon phosphorImager).

Support Protocol 1: GENERATION OF S10 HeLa EXTRACTS FOR IVTR ASSAY

It is good practice to prepare all buffers fresh the night before the experiment. Chill the isotonic buffer, hypotonic buffer, dialysis buffer I, dounce homogenizer and pestle overnight at 4°C.

Materials

HeLa S3 cells (Unit 15H.1, Support Protocol 3)

Fetal Bovine Serum (FBS)

Isotonic Buffer

Hypotonic Buffer

40 mL capacity glass dounce homogenizer with tight pestle B (Bellco)

10× Extract Buffer

Dialysis Buffer I

5 mL capacity Slide-A-Lyzer Cassette 10,000 MWCO (ThermoScientific)

1 M $CaCl_2$

150 U/µL S7 Micrococcal Nuclease (Roche) in 1 mM Tris

1 M EGTA

1. Grow 3 L of HeLa S3 suspension culture at ~5×10^5 cells/mL (Unit 15H.1, Support Protocol 3).

   The night before harvesting: add an additional 100mL FBS to 3 L of cells. This gives the cells an extra boost.

2. Harvest the entire HeLa S3 suspension culture by pouring the cells equally into six 500 mL conical tubes. To pellet cells, centrifuge 15 min at 200× *g*.

   The suspension culture should contain approximately 4–6 $\times10^5$ cells/mL.

3. Remove supernatant by carefully pouring off medium.

   It is important to work quickly and carefully during this step to avoid dislodging the cell pellet.

4. Resuspend and combine the six cell pellets in a total volume of 40 mL of ice-cold isotonic buffer and transfer to a 50 mL conical tube.

5. Centrifuge 5 min at 200× *g*. Remove the supernatant.

6. Wash the cells by adding 30 mL of ice-cold isotonic buffer and pipetting up and down. Centrifuge 5 min at 200× *g*. Remove supernatant.

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7. Repeat step 6 once.
8. Resuspend pellet in 1 volume of ice-cold hypotonic buffer (approximately 5 mL). Incubate 10 min on ice.
9. Transfer suspension into 40 mL capacity glass dounce homogenizer.

   Dounce homogenizer should be stored at 4ºC overnight before use.

10. Using the tight pestle B, dounce 25 strokes (straight down and strong) into the homogenizer.
11. Immediately add 0.1 volume of 10× extract buffer and transfer contents of homogenizer into a 14 mL culture tube.
12. Centrifuge 10 min at 500× *g*, 4ºC.
13. Separate the supernatant from the pellet fraction and place it into a new 14 mL culture tube.
14. Centrifuge both tubes 10 min at 10,000× *g*, 4ºC.
15. Combine supernatant of both tubes (approximately 3–4 mL) and transfer into a hydrated 10,000 MWCO Slide-A-Lyzer cassette.

    Hydrate the cassette in dialysis buffer I for at least two minutes prior to adding extract. It is important to not pierce the dialysis membrane while adding the supernatant. Also, be sure to remove all air bubbles prior to dialysis.

16. Fill a 2 L beaker with 1.5 L of chilled dialysis buffer I. Float cassette with extract in the buffer. Dialyze 1 h at 4ºC.
17. Replace the dialysis buffer I with fresh buffer. Dialyze 1 h at 4ºC.
18. Remove extract from dialysis cassette and add 1/1000 volume 1 M $CaCl_2$ and 75 U (0.5 μL) per mL S7 Micrococcal Nuclease. Incubate 15 min at room temperature.
19. Add 1/100 volume of 200 mM EGTA. Centrifuge 10 min at 10,000× *g*, 4ºC.

    Store at −70ºC. It is best to freeze aliquots of 100, 250 and 500 μL to avoid multiple freeze thaw cycles.

Support Protocol 2: GENERATION OF INITIATION FACTORS FOR IVTR ASSAY

Initiation factors (IF) from HeLa S3 cell extracts are necessary for efficient translation of poliovirus RNA *in vitro*. Furthermore, it has been shown that higher viral titers can be reached by adding IF to *in vitro* translation reactions (Barton et al., 1995). It is good practice to prepare all buffers fresh the night before the experiment. Chill the isotonic buffer, hypotonic buffer, dialysis buffer II, dounce homogenizer and pestle overnight at 4°C.

Materials

HeLa S3 cells (Unit 15H.1, Support Protocol 3)

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Fetal Bovine Serum (FBS)

Isotonic Buffer

Hypotonic Buffer

40 mL capacity glass dounce homogenizer with tight pestle B (Bellco)

Dialysis Buffer II

5 mL capacity Slide-A-Lyzer Cassette 10,000 MWCO (ThermoScientific)

4 M KCl

Ice

25 × 38 mm Quick-Seal Polyallomar Tubes (Beckman #343664)

Beckman 70 Ti rotor

1. Grow 3 L of HeLa S3 suspension culture at ~5×10^5 cells/mL (Unit 15H.1, Support Protocol 3).

   The night before harvesting: add an additional 100mL FBS to 3 L of cells. This gives the cells an extra boost.

2. Harvest the entire HeLa S3 suspension culture by pouring the cells equally into six 500 mL conical tubes. Centrifuge cells 15 min at 200× *g*.

   Suspension culture should contain approximately 4–6 $\times10^5$ cells/mL.

3. Remove supernatant by carefully pouring off medium.

   It is important to work quickly and carefully during this step to avoid dislodging the cell pellet.

4. Resuspend and combine the six cell pellets in a total volume of 40 mL of ice-cold isotonic buffer and transfer to a 50 mL conical tube.

5. Centrifuge 5 min at 200× *g*. Remove the supernatant.

6. Wash the cells by adding 30 mL of ice-cold isotonic buffer and pipetting up and down. Centrifuge 5 min at 200× *g*. Remove supernatant.

7. Repeat step 6 once.

8. Resuspend pellet in 1 volume of ice-cold hypotonic buffer (approximately 5 mL). Incubate 10 min on ice.

9. Transfer suspension into 40 mL capacity glass dounce homogenizer.

   Dounce homogenizer should be stored at 4°C overnight before use.

10. Using the tight pestle B, dounce 25 strokes (straight down and strong) into the homogenizer.

11. Transfer extract to a 14 mL culture tube. To remove nuclei, centrifuge 10 min at 500× *g*, 4°C.

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12. Transfer the post-nuclear supernatant into a fresh tube and centrifuge 15 min at 12,000× *g*, 4°C.

    We generally use a 25 × 38 mm Quick-Seal Polyallomar Tube in a Beckman centrifuge with a JA-20 rotor at 10,000 rpm.

13. Transfer supernatant to a clean 25 × 38 mm Quick-Seal Polyallomar Tube. To pellet ribosomes, centrifuge supernatant 1 h at 60,000 rpm, 4°C in 70 Ti rotor (Beckman).

14. Discard supernatant and resuspend the ribosomal pellet in hypotonic buffer at a concentration of 240 A_{260} U/mL.

15. Adjust the concentration of KCl in the ribosomal pellet by adding 4 M KCl to a final concentration of 0.5 M. Stir for 15 minutes on ice.

16. Centrifuge the salt-washed ribosomes 1 h at 60,000 rpm, 4°C in 70 Ti rotor.

17. Transfer the supernatant into a hydrated 10,000 MWCO Slide-A-Lyzer cassette.

    Hydrate the cassette in dialysis buffer II for at least two minutes prior to adding extract. It is important to not pierce the dialysis membrane while adding the supernatant. Also, be sure to remove all air bubbles prior to dialysis.

18. Fill a 2 L beaker with 1.5 L of dialysis buffer II. Float cassette with extract in the buffer. Dialyze 4 h at 4°C.

19. Transfer extract containing the initiation factors to 1.5 mL tubes for storage.

    Store at −70°C. It is best to freeze aliquots of 10, 25 and 50 μL to avoid multiple freeze thaw cycles of the initiation factors.

Basic Protocol 6: ASSAYING REPLICATION BY STRAND-SPECIFIC qPCR

Similar to a one-step growth curve (Basic Protocol 2), which measures the production of viable virions over time, this protocol measures the production of +strand genomes and −strand templates over time. It hinges on the use of *in vitro* transcribed PV RNA (see Unit 15H.1, Basic Protocol 1) as a quantifiable standard. A derivative of prib(+)XpA (Herold and Andino, 2000), which contains an inverted T7 promoter 3′ to the polyA tail allows transcription of both +strand and −strand PV RNA. This plasmid, prib(+)XpA+or-, can be linearized for +strand *in vitro* transcription (IVT) using *Eco*RI and linearized for −strand *in vitro* transcription using *Sma*I. Dilution series of standard RNAs of each sense are run alongside experimental samples in order to measure the precise number of copies in each sample. For the method to be quantitative, the linear range of the reverse transcription and qPCR reactions must be considered as noted in the protocol. Also, it is important for quantitation that pipet calibration be up to date. The strand-specific nature of this protocol is based on a method described in Plaskon et al., 2009.

Our suggested protocol is to perform infections in biological triplicates and harvest samples every hour from 1 to 8 h post-infection. Unless the infections are performed at a very high MOI (~100), −strand is unlikely to be detectable until two hours post infection. We suggest performing the infection with sucrose cushion purified virus (Unit 15H.1, Basic Protocol 5).

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If the infecting virus has not been purified via sucrose cushion purification, a significant quantity of unpackaged RNA (from the generation of the stock) will be present in the inoculum. A large portion of this RNA will stick to the cells, despite the washes, resulting in a low signal to noise ratio for the first two hours of the infection until *de novo* RNA synthesis has occurred.

Materials

HeLa or HeLa S3 cells (Unit 15H.1, Support Protocol 2)

10% NCS DMEM/F-12

P0 or other infecting stocks of known titer

Serum-free DMEM/F-12

PBS

2.5% NCS DMEM/F-12

Purelink RNA Mini kit (Life Technologies)

2-mercaptoethanol

70% (v/v) EtOH

Nuclease-free water

5 μM RT primers (+strand_RT, −strand_RT; Table 1)

10 mM dNTP Mix

200 U/μL SuperScript III RT (Life Technologies)

5× First-Synthesis Buffer (provided with SuperScript III RT)

0.1 M DTT (provided with SuperScript III RT)

40 U/μL RNaseOUT (Life Technologies)

10 U/μL ExonucleaseI (Fermentas)

10× ExonucleaseI buffer (provided with ExonucleaseI)

2× Fast Mastermix (KAPA Biosystems)

10 μM PCR primers (+strand_F, −strand_R, Tag; Table 1)

37°C and 5% CO_2 humidified incubator

Ice

20 μL multichannel pipette

Perform infection

1. Seed HeLa or HeLa S3 cells in 24-well plates at 2.5×10^5 cells in 500 μL of 10% NCS DMEM/F12 per well and incubate 24 h at 37°C in 5% CO_2, at which time

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they should be nearly confluent. Prepare 3 replicate wells per sample. Prepare 1 plate for each time point.

If a precise MOI is desired, seed an additional 4 wells of cells for counting.

2. Thaw infecting stock(s) and dilute to 1×10^8 PFU/mL in serum-free DMEM/F-12. Always change tips when performing serial dilutions. Allow 50 μL inoculum per well. Keep inoculum on ice.

   If a precise MOI is desired, trypsinize and count 4 wells of cells and adjust the inoculum concentration accordingly for an MOI of 10.

3. Remove plates from incubator and place on ice. Aspirate medium.
4. Wash cells with 500 μL cold serum-free DMEM/F-12 per well.
5. Aspirate serum-free DMEM/F-12 and add 50 μL cold inoculum per well.
6. Incubate plates 30 min at 4ºC. Rock plates to distribute inoculum every 5–10 min.
7. Return plates to ice and aspirate inoculum. Wash cells twice with 500 μL cold serum-free DMEM/F-12 per well.
8. Add 100 μL warm (37ºC) 2.5% NCS DMEM/F12 per well.
9. Incubate plates at 37ºC in 5% CO_2. This is time = 0.
10. At each time point, transfer one plate from 37ºC to −70ºC.

Extract RNA

11. Remove plates from −70ºC. Before cell lysate thaws completely, add 600 μL Cell Lysis buffer from Purelink RNA Mini kit (with 2-mercaptoethanol added to 1%).
12. Using a P1000 pipet, pipet up and down 10× to lyse cells. Transfer to a 1.5 mL tube.
13. Add 700 μL 70% EtOH and vortex 10 seconds to mix.
14. Follow remaining instructions in Purelink RNA Micro kit.
15. Elute in 30 μL nuclease-free water.

    Store RNA at −70ºC.

Synthesize cDNA

16. Perform cDNA synthesis in separate reactions for +strand and −strand for each sample:

    Always work with RNA on ice.

    a. Prepare a master mix for sample denaturation. Prepare 10% extra mix to ensure enough mix for all reactions.

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0.625 µL 10 mM dNTP mix

0.125 µL 5 µM RT primer

0.25 µL nuclease-free water

For +strand cDNA synthesis, use "+strand_RT" RT primer. For −strand cDNA synthesis, use "-strand_RT" RT primer.

b. For each sample add 1 µL sample denaturation mix to 2.5 µL RNA. Incubate 5 min at 95°C, then 5 min at 70°C, then place on ice.

For standard RNAs, use five 10-fold dilutions of IVT RNA of each sense starting with 2.4 $\times 10^7$ copies/µL (95.6 pg/µL). The upper limit of the linear range of this reaction is 6 $\times 10^7$ copies/reaction. Therefore, most experimental RNA samples must be diluted before being reverse transcribed. The following suggested dilutions are suggested for samples from MOI =10 infections of 5 $\times 10^5$ cells:

+strand: 0–2 h 1/10; 3–4 h 1/1000; 5–8 h 1/10,000

−strand: 4–8 h 1/1000

c. Prepare a master mix for cDNA synthesis. Prepare 10% extra mix to ensure enough mix for all reactions.

1.0 µL 5× First-Strand synthesis buffer

0.25 µL 0.1 M DTT

0.125 µL 200 U/µL SuperScript III RT

0.125 µL 40 U/µL RNaseOUT

d. Add 1.5 µL cDNA synthesis mix to each tube. Incubate 30 min at 55°C, then 15 min at 70°C.

Store cDNA at −20°C.

Exonuclease treat cDNA

Exonuclease treatment of RT products is necessary to ensure leftover RT primers cannot participate in the following PCR reaction.

17 Prepare a master mix for exonuclease treatment. Prepare 10% extra mix to ensure enough mix for all reactions.

0.05 µL 10 U/µL ExonucleaseI

0.375 µL 10× ExonucleaseI buffer

0.575 µL nuclease-free water

18 Add 1 µL exonuclease mix to each tube. Incubate 30 min at 37°C, then 15 min at 80°C.

Store cDNA at −20ºC.

Perform qPCR

19. Prepare a master mix for qPCR. Prepare 10% extra mix to ensure enough mix for all reactions.

    5.0 μL 2× Fast Mastermix

    0.2 μL 10 μM For primer

    0.2 μL 10 μM Rev primer

    0.6 μL water

    Perform technical duplicates or triplicates of each reaction which can be averaged to ensure proper quantification of each sample. For +strand qPCR, use "+strand_F" and "Tag." For −strand qPCR, use "Tag" and "-strand_R."

20. Using a multichannel pipet, add 6 μL PCR mix to each well.

21. Using a multichannel pipet, add 4 μL diluted RT products to each well.

    The upper limit of the linear range of this reaction is 1×10^6 copies/reaction. Therefore, all RNA samples must be diluted 1/40 before added to the PCR reaction. This is most easily accomplished by adding 234 μL water to each sample in an 8-strip tube using a multichannel pipet.

22. Perform qPCR cycles (below) and analyze data.

First step:	5:00	95ºC	(initial denaturation)
40 cycles:	0:05	95ºC	(denaturation)
	0:20	60ºC	(annealing-extension)

Basic Protocol 7: IMMUNOPRECIPITATION OF PV VIRIONS

This method is a quick, but relatively small-scale, purification that uses magnetic beads bound to anti-poliovirus antibodies to isolate PV virions. It can be used to quantify the amount of packaged PV RNA in experimental samples or simply as a quick way to obtain purified PV RNA for downstream applications. In conjunction with Basic Protocol 6, this method allows quantitative, temporal analysis of PV encapsidation. The method is based on Life Technologies' general guidelines for immunoprecipitation.

Materials

Experimental lysates (such as those generated in step 11 of Basic Protocol 2)

Dynabeads Protein A (Life Technologies)

DynaMag-2 magnet (Life Technologies)

1 μg/μL anti-poliovirus type 1 monoclonal antibody (14D2, Pierce Biotechnology)

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PBS + 0.02% (v/v) Tween-20

PBS

50 mM Glycine (pH 2.8)

1 M Tris-HCl (pH 7.5)

1 mg/mL RnaseA (Life Technologies)

Purelink RNA Micro kit (Life Technologies)

Prepare Dynabeads

1 Resuspend Dynabeads in the vial (vortex >30 s or tilt and rotate 5 min).

2 Transfer 5 μL beads per sample to a 1.5 mL tube.

3 Place the tube on the magnet for 1 min. Remove supernatant.

4 Remove the tube from the magnet.

Bind antibody to Dynabeads

5 Dilute 1 μL antibody per sample in a total volume of 200 μL PBS + 0.02% (v/v) Tween-20. Add diluted antibody to Dynabeads.

6 To allow Dynabead-antibody complex to form, incubate tube with rotation 10–20 min at room temperature.

7 While antibody and beads are incubating, prepare 1 tube per sample with 20 μL experimental lysate.

8 Add 70 μL PBS to each tube containing lysate.

9 Place the tube containing the Dynabeads on the magnet for 1 min. Remove supernatant.

10 Remove the tube from the magnet.

11 Resuspend Dynabeads in 200 μL PBS with 0.02% Tween-20 by gentle vortex.

This wash removes excess unbound antibody.

12 Place the tube on the magnet for 1 min. Remove supernatant.

13 Remove the tube from the magnet.

14 Resuspend Dynabeads in PBS to twice the volume of beads that you started with by gentle vortex.

If co-elution of the antibody is a problem for downstream applications, cross-linking of the antibody to the beads must be performed at this point. Cross-linking reagents are available from Life Technologies.

Bind capsids to antibody

15 Aliquot 10 μL Dynabeads into each tube containing experimental lysate.

Gently vortex between samples to ensure even distribution of beads.

16. Mix by gentle vortex.

17. To allow antibody-capsid complex to form, incubate tubes with rotation 10–20 min at room temperature.

18. Place the tubes on the magnet for 1 min. Remove supernatant.

19. Remove the tubes from the magnet.

20. Resuspend Dynabeads in 200 μL PBS by gentle vortex.

21. Place the tubes on the magnet for 1 min. Remove supernatant.

22. Remove the tubes from the magnet.

23. Repeat steps 20–22 twice.

    These washes remove unbound proteins and nucleic acids.

24. Resuspend Dynabeads in 100 μL PBS and transfer to a clean 1.5 mL tube.

    Transferring to a clean tube avoids co-elution of proteins that have nonspecifically bound to the walls of the tube.

Elute capsids from Dynabeads

25. Place the tubes on the magnet for 1 min. Remove supernatant.

26. Remove the tubes from the magnet.

27. Add 20 μL 50 mM Glycine (pH 2.8) and mix by gentle vortex.

28. Incubate with rotation 2 min at room temperature.

29. Place the tubes on the magnet for 1 min.

30. Transfer 20 μL supernatant (eluate) to a new tube.

RNaseA treat eluate

31. Add 20 μL 1 M Tris-HCl (pH 7.5) and 0.4 μL 1 mg/mL RNaseA to eluate.

32. Incubate 30 min at 37ºC.

    RNaseA treatment of the eluate removes unencapsidated RNA that nonspecifically bound to the Dynabead-antibody complex. Store at –20ºC.

Purify RNA for qRT-PCR or other downstream applications

33. Follow instructions in Purelink RNA Micro kit, being sure to include carrier RNA during purification.

34. Elute in 10 μL nuclease-free water.

    Store RNA at –70ºC.

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Basic Protocol 8: ASSAYING TRANSLATION BY WESTERN BLOT

The generation of monoclonal and polyclonal antibodies against the nonstructural proteins of PV has made it possible to observe the expression of the viral proteins during the course of replication. This is especially useful when analyzing mutant viruses.

Materials

Virus Stock(s) to be analyzed

HeLa S3 cells (Unit 15H.1, Support Protocol 2)

PBS

0.05% Trypsin-EDTA

10% NCS DMEM/F-12

6-well plates

0.5% NP-40 Lysis Buffer

2× protein loading dye

Equipment for Western blot (Gallagher, 2010)

Ice

37ºC and 5% CO_2 humidified incubator

Prepare and infect cells

1 Seed HeLa S3 cells in 6-well plates at 1.0×10^6 cells in 2 mL of 10% NCS DMEM/F-12 per well and incubate 24 h at 37ºC in 5% CO_2.

If performing a time course of protein expression during viral infection, prepare one well for each time point. A separate plate should be used for each time point to avoid repeatedly removing samples from the incubator.

2 On the day of the assay, thaw virus stocks and dilute to 1.6×10^8 PFU/mL in 10% NCS DMEM/F-12. You will need 250 μL inoculum per time point plus 5–10% extra to account for pipetting error.

This concentration gives a multiplicity of infection (MOI) of ~20. A confluent well has ~ 2×10^6 cells. For an MOI of ~20, you need ~4×10^7 PFU/0.25 mL inoculum = ~ 1.6×10^8 PFU/mL.

3 Aspirate medium and wash cells once with 1 mL/well of PBS. Aspirate PBS.

4 Infect cells with 250 μL of viral inoculum. Rock plates back and forth to evenly spread the inoculum. Incubate plates 30 min at 37ºC and 5% CO_2.

During the incubation period, return to the incubator to rock plates back and forth every 10 minutes. This ensures the virus is spread evenly and has an opportunity to attach to all the cells.

5. Aspirate viral inoculum.

6. Wash cells by adding 1 mL/well of PBS and rocking plate back and forth. Aspirate PBS.

7. Repeat step 5 twice.

8. Add 1 mL/well 10% NCS DMEM/F-12. Incubate at 37°C and 5% CO_2.

9. Collect samples at desired time post-infection.

   We generally collect samples between 1 and 8 h post-infection (one infectious cycle).

Collect samples

10. Using a cell scraper, gently remove cells from the plate and transfer to a 1.5 mL tube.

11. To pellet the cells, centrifuge 1 min at 500× *g*. Carefully remove supernatant and resuspend cells in 50 uL 0.5% NP-40 Lysis Buffer. Incubate 30 min on ice.

    Incubation times can range from 20 min to 2 h. It is good practice to observe cells under a light microscope to verify lysis has occurred.

12. To pellet cellular debris, centrifuge 1 min at 12,000× *g*. Transfer supernatant to a new 1.5 mL tube.

    Store cell lysates at −20°C or proceed with SDS-PAGE and Western Blot analysis.

Perform SDS-PAGE and Western blot analysis

13. Combine 5 μL of cell lysate with 5 μL of 2× protein loading buffer.

14. Perform SDS-PAGE, transfer proteins to membrane, probe blots for viral proteins and visualize bands according to standard Western blot protocols (Gallagher, 2010).

    We use the following dilutions for PV antibodies:

    α -2C (Crotty et al., 2004): 1:15,000

    α -3A (Doedens et al., 1997): 1:125

    α -3D (Bienz et al., 1990): 1:20,000

    Antibodies for the poliovirus capsid are commercially available.

15. Continuing to follow standard protocols (Gallagher, 2010), strip membrane and re-probe with an internal loading control (e.g. α-Actin or α-GAPDH).

    This ensures that roughly equal amounts of cell lysate were loaded on the gel. If the amounts of proteins are drastically different between samples, it is possible that the number of cells seeded per well prior to infection was not equal.

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Basic Protocol 9: ASSAYING TRANSLATION BY ^{35}S-MET LABELING

This assay is useful for detecting major changes in protein synthesis. Because PV shuts down host cell translation (reviewed in (Kuechler et al., 2002; Zamora et al., 2002)), straightforward metabolic labeling of proteins starting at 4–5 h post-infection achieves labeling of viral proteins only, with little cellular protein background.

Materials

Virus stocks of known titer to be assayed

HeLa or HeLa S3 cells (Unit 15H.1, Support Protocol 2)

10% NCS DMEM/F-12

Serum-free DMEM/F-12

PBS

PBS + 1% (v/v) Triton X-100

Methionine- and cysteine-free DMEM (Life Technologies 21013-024)

EasyTag EXPRESS^{35}S Protein Labeling Mix (PerkinElmer)

NENSure charcoal trap (PerkinElmer)

Charcoal paper

2× protein loading dye

SDS-PAGE gels and running buffer (Manns, 2011)

10% (v/v) glycerol

6-well plates

37ºC and 5% CO_2 humidified incubator

Prepare cells, virus, and labeling medium

1. Seed HeLa or HeLa S3 cells in a 6-well plate at 1×10^6 cells in 2 mL of 10% NCS DMEM/F-12 per well and incubate 24 h at 37ºC in 5% CO_2, at which time they should be confluent. If assaying more than 6 viruses, prepare extra plates (allow 1 well/virus).

2. Prepare labeling medium by supplementing methionine- and cysteine-free DMEM with 100 μCi/mL EasyTag EXPRESS^{35}S Protein Labeling Mix. You will need 1 mL per virus plus 5–10% extra to account for pipetting error.

   Before opening vial of Labeling Mix, vent with NENSure charcoal trap. Labeling medium may be stored at 4ºC overnight.

3. On the day of the assay, thaw virus stocks and dilute to 2×10^8 PFU/mL in cold serum-free DMEM/F-12. You will need 0.5 mL inoculum per virus plus 5–10% extra to account for pipetting error.

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This concentration gives a multiplicity of infection (MOI) of ~50. A confluent well has ~ 2×10^6 cells. For an MOI of ~50, you need ~1×10^8 PFU/0.5 mL inoculum = ~ 2×10^8 PFU/mL. A high MOI is helpful to ensure rapid and complete shutoff of host cell translation, but if stock titers are too low it can be reduced.

Infect and label cells

4 Aspirate old medium and wash cells with 2 mL/well PBS.

5 Aspirate PBS and cover cells with 0.5 mL viral inoculum (one virus per well).

6 Incubate 30 min at 37ºC and 5% CO_2.

7 Aspirate inoculum and cover cells with 1 mL serum-free DMEM/F-12.

8 Incubate 2 h at 37ºC and 5% CO_2.

9 Aspirate medium and wash cells twice with 2 mL/well PBS. Cover cells with 1 mL/well methionine- and cysteine-free DMEM.

10 Incubate 2 h at 37ºC and 5% CO_2.

11 Aspirate medium and cover cells with 1 mL/well labeling medium.

12 Incubate 2 h at 37ºC and 5% CO_2.

Once radioactivity is added, plates should be contained within the incubator in a box lined with charcoal paper. ^{35}S-Methionine is volatile, and the charcoal paper serves to absorb any aerosolized radioactivity. Be sure to dispose of all waste from this point forward according to both Biological and Radioactive safety guidelines.

13 Aspirate medium and wash cells twice with 2 mL/well PBS. Cover cells with 1 mL/well PBS and scrape into 1.5 mL tube using a cell scraper or pipet tip.

14 To gently pellet cells, centrifuge at 1000× *g* for 2 min.

15 Aspirate PBS and resuspend cells in 100 μL PBS + 1% Triton X-100.

16 To pellet cellular debris, centrifuge at 12,000× *g* for 2 min.

17 Transfer supernatant to clean 1.5 mL tube.

Store at −20ºC.

Analyze Proteins by SDS-PAGE

18 Combine 10 μL labeled supernatant with 10 μL 2× protein loading dye. Incubate 5 min at 95ºC.

19 Load onto SDS-PAGE gel (10% resolving/5% stacking works well) and run at 250 V until dye migrates off (Manns, 2011).

20 Soak gel in 10% glycerol for 30 min.

21 Dry gel at 85ºC for 4 h.

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22. Expose to film through plastic wrap overnight.
23. Develop gel and observe proteins.
24. If protein bands are not clearly visible, repeat step 22 with longer exposure (up to 48 h).

REAGENTS AND SOLUTIONS

Cell culture medium and supplements

DMEM/F-12 is used for monolayer cultures. It is a 1:1 mixture of Dulbecco's Modified Eagle Medium and Ham's F-12. It is available commercially from several companies. We purchase 450 mL bottles for most purposes. Like most tissue culture medium, these contain L-glutamine, which breaks down in solution over time. One approach is to supplement each bottle with fresh L-glutamine, as we do. There are also commercially available media that use a more stable form, such as a glutamine dipeptide, which obviates the need for such supplementation. It is also possible to supplement with antibiotics and anti-fungals, to reduce contamination. We generally supplement with a 100× mixture of penicillin, streptomycin, and L-glutamine, which is commercially available. Newborn Calf Serum (NCS) is cheaper than Fetal Bovine Serum (FBS) and more than adequate for HeLa and HeLa S3 monolayer growth.

Serum-free DMEM/F-12

450 mL DMEM/F-12 medium

4.5 mL 100× penicillin-streptomycin-glutamine

Store at 4°C for 1 month.

2.5% NCS DMEM/F-12

450 mL DMEM/F12 medium

11.5 mL Newborn Calf Serum

4.6 mL 100× penicillin-streptomycin-glutamine

Store at 4°C for 1 month.

10% NCS DMEM/F-12

450 mL DMEM/F-12 medium

50 mL Newborn Calf Serum

5.0 mL 100× penicillin-streptomycin-glutamine

Store at 4°C for 1 month.

Dialysis Buffer I

40 mM HEPES (pH 8.0)

120 mM KOAc

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5.5 mM MgOAc

10 mM KCl

6 mM DTT

Store at 4°C for 12 months.

Dialysis Buffer II

5 mM Tris-HCl (pH 7.5)

100 mM KCl

0.05 mM EDTA

1 mM DTT

5% (v/v) glycerol

Store at 4°C for 12 months.

10× Extract Buffer

200 mM HEPES (pH 7.4)

1200 mM KOAc

40 mM MgOAc

50 mM DTT

Store at room temperature for 12 months.

Hypotonic Buffer

20 mM HEPES (pH 7.4)

10 mM KCl

1.5 mM MgOAc

1 mM DTT

Store at room temperature for 12 months.

Isotonic Buffer

35 mM HEPES (pH 7.4)

146 mM NaCl

11 mM Glucose

Store at 4°C for 12 months.

10× NTP-Rx Buffer without UTP

10 mM ATP

2.5 mM CTP

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2.5 mM GTP

600 mM KOAc

300 mM Creatine Phosphate

155 mM HEPES (pH 7.0)

Store at room temperature for 12 months.

Immediately before use, add 0.4 mg Creatine Kinase to 100 μL of 10× NTP-Rx Buffer without UTP.

TENSK Buffer

50 mM Tris (pH 7.6)

5 mM EDTA

100 mM NaCl

1% (w/v) SDS

Store at room temperature for 12 months.

Immediately before use, add 1 mg Proteinase K to 1 mL of TENS Buffer.

0.5% NP-40 Lysis Buffer

0.5% (v/v) NP-40

150 mM NaCl

50 mM Tris-HCl (pH 7.5)

Store at 4°C for 12 months.

COMMENTARY

Background Information

Poliovirus (PV) was identified as the causative agent of paralytic poliomyelitis in 1909. The disease reached epidemic proportions in the United States in the early twentieth century and remained so until the development of first the inactivated Salk vaccine in 1952 and then the live attenuated Sabin vaccine in 1963. Disease incidence in the developed world has plummeted, and since 1988 it has been the target of a global WHO eradication campaign. As of 2012, PV is endemic to only three countries: Afghanistan, Nigeria, and Pakistan. [For a review of the history of poliomyelitis, see (De Jesus, 2007).]

The first full length genome sequences and infectious cDNA clones of PV were produced in 1981 (Kitamura et al., 1981; Racaniello and Baltimore, 1981). Humans are the only natural host for PV infections, but other primates can also be infected. In tissue culture, PV infects a wide range of human and non-human primate cell lines. The factor limiting host range is expression of the poliovirus receptor (PVR or CD155), and transgenic mice and mouse cell lines that express the receptor are available (Mendelsohn et al., 1989; Ren et al., 1990).

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The poliovirus genome is 7440 nucleotides long, excluding a poly(A) tail of a variable length at the 3′ end. It can be divided into a 742-nucleotide 5′ untranslated region (5′UTR), a single long open reading frame encoding the viral polyprotein, and a 68-nucleotide 3′ untranslated region (3′UTR). A small viral protein of 22 amino acids, VPg (3B), is covalently attached to the 5′ end of the genome. The mature virion consists of an icosahedral protein shell, composed of four capsid proteins (VP1, VP2, VP3, and VP4), which encapsidates the genome (Hogle et al., 1985). The viral genome serves as an mRNA (which is why transfection of *in vitro* transcribed genomes produces virus), and the entire viral life cycle takes place in the cytoplasm, with replication occurring on specialized membraneous structures derived from the host cell organelles. A full replication cycle in HeLa or HeLa S3 cell is approximately 8 h.

Poliovirus mutants have been useful at many stages in the history of mammalian virology, and many assays have been developed to characterize the effect of mutations on different parts of the PV life cycle, most specifically viral translation and replication.

Critical Parameters and Troubleshooting

Cloning—Working with the large poliovirus cDNA constructs can sometimes be challenging as many standard cell lines and cloning methods don't work well. For some reason, the 3′ portion of the genome (specifically the region encoding 3D) is particularly difficult. A dearth of restriction sites exacerbates the problem, since it requires you to work with very large inserts. The protocol suggested here (Basic Protocol 1) is a combination of mostly successful techniques based on much trial and error.

Tissue culture problems—The most common problem is contamination with bacteria or fungi. This can be reduced by the use of careful aseptic technique. Work should always be performed in a biosafety cabinet, and surfaces and supplies wiped down with 75% ethanol. Routine use of penicillin and streptomycin in culture medium also reduces bacterial contamination. Also, if tissue culture work and bacterial work (e.g. Basic Protocol 1) are to be performed on the same day, always do the tissue culture work first.

Anticipated Results

Viral replication is an exponential growth process, so results from Basic Protocols 2–4 and 6 are typically spread over several orders of magnitude. Therefore, it is useful to use a logarithmic scale graph to display them. Severe mutants will often differ from wild type by an order of magnitude or more. For typical results of a one-step growth curve (Basic Protocol 2) or drug resistance curve (Basic Protocol 3), see (Vignuzzi et al., 2008). For typical results of a replicon assay (Basic Protocol 4) or IVTR assay (Basic Protocol 5), see (Vogt and Andino, 2010). For typical results of Basic Protocols 6–8, see Burrill et al., in preparation. For typical results of protein radiolabeling (Basic Protocol 9), see (Lauring et al., 2012).

Time Considerations

Starting with a good prep of the relevant vector, cloning a new mutant (Basic Protocol 1) takes a minimum of 5 days: a day for the two overlap PCR reactions, another day for the

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digestions, ligation, and transformation, a third day to start the mini-cultures, a fourth to prep and sequence them, and a fifth to analyze the sequencing results. It will then take another 10 days to 2 weeks to generate and titer a P0 virus stock (see Unit 15H.1).

Basic Protocols 2 and 3 each take only 24 hours to perform, but require another 1–2 weeks to titer the samples (again, see Unit 15H.1). Replicon experiments (Basic Protocol 4) typically take 8 hours, with another 1–2 hours to assay luciferase activity. The IVTR assay (Basic Protocol 5) takes 7 hours to perform, and another ~2 hours for gel electrophoresis, followed by up to 24 hours for autoradiography development. Basic Protocol 6 requires a day for the infection and sampling, and another 1–2 days for RNA extraction, RT, and qPCR. Immunoprecipitation (Basic Protocol 7) requires approximately half a day. Basic Protocol 8 requires a day for the infection and sampling, and another 4 hours for Western Blot analysis. ^{35}S-Met labeling of proteins typically takes a full day (with several 2 h incubations) and part of a second day to analyze the proteins by SDS-PAGE (Basic Protocol 9).

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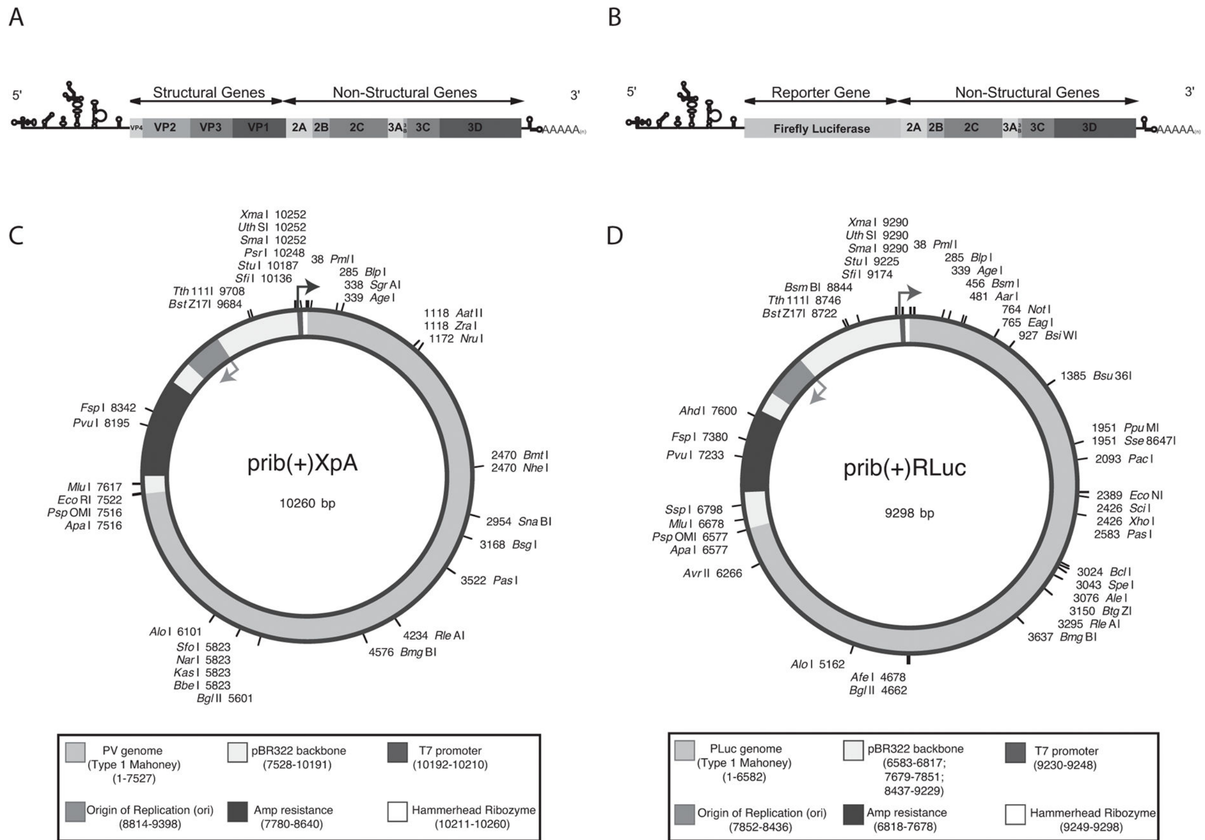

Figure 1.

(A) Schematic of the PV Genome. The PV genome includes a highly structured 5′UTR followed by a single open reading frame and a polyadenylated 3′UTR. The coding region can be separated into structural and non-structural genes. The structural genes are required to form the viral capsid and the non-structural genes are all required for a successful replication cycle. **(B)** Schematic of the PLuc Genome. The PLuc genome contains a Firefly Luciferase reporter gene followed by the non-strucutural genes required for replication. The open reading frame is flanked by a highly structured 5′ UTR and a polyadenylated 3′UTR. **(C)** prib(+)XpA plasmid map. PV (Mahoney) genome is in the pBR322 backbone. The plasmid contains an ampicillin resistance casette, a T7 promoter, and a hammerhead ribozyme. Location of single cut restriction sites are shown. **(D)** prib(+)RLuc plasmid map. PLuc genome is in the pBR322 backbone. The plasmid contains an ampicillin resistance casette, a T7 promoter, and a hammerhead ribozyme. Location of single cut restriction sites are shown.

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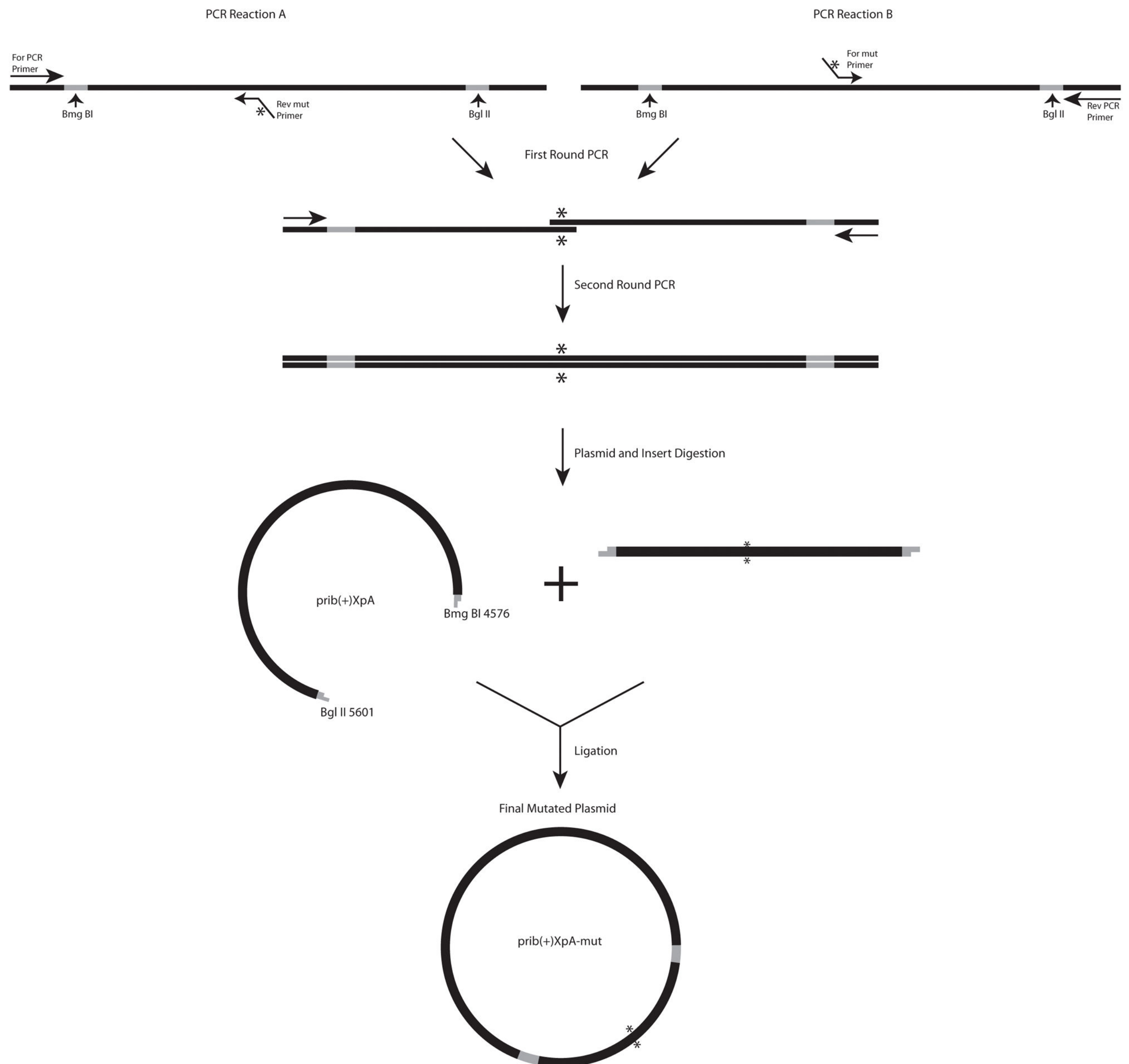

Figure 2.
Mutagenesis of cDNA plasmid by overlap PCR and restriction cloning. Two first round PCR reactions are performed using prib(+)XpA plasmid as a template and primers with gene specific and mutated sequence. A second round of PCR is then performed using the products from the two first round PCR Reactions A and B as a template with the gene specific primers. Both the plasmid and insert are digested then ligated to create the final mutated plasmid.

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Table 1

qPCR Primers

Primer Name	Sequence
+strand_RT	GGCCGTCATGGTGGCGAATAAtgtgatggatccgggggtagcg
−strand_RT	GGCCGTCATGGTGGCGAATAAcatggcagccccggaacagg
+strand_F	catggcagccccggaacagg
−strand_R	tgtgatggatccgggggtagcg
Tag	GGCCGTCATGGTGGCGAATAA