

Construction and Isolation of Recombinant Vaccinia Virus Using Genetic Markers

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Summary

The standard approach for the isolation of vaccinia virus recombinants involves homologous recombination between a transfected plasmid and the replicating viral DNA. In a typical infection/transfection experiment, recombinant viruses only account for a tiny proportion (10^{-4} to 10^{-3}) of the progeny virus; thus, genetic markers are often included in the transfected plasmid to facilitate the selection of recombinant viruses. This chapter describes in detail two different selection procedures: one relies on plaque formation phenotype using the vaccinia virus gene F13L; the other relies on antibiotic resistance using the *Escherichia coli* xanthine–guanine phosphoribosyl transferase gene.

Key Words: Recombinant vaccinia virus; plaque size; F13L; xanthine–guanine phosphoribosyl transferase; *gpt*.

1. Introduction

Procedures for the genetic modification of poxviruses must take into account several aspects of their biology, particularly, the size of the viral genome and that naked poxvirus DNA is not infectious. As a general rule, recombinant vaccinia viruses are generated inside cells by recombination between transfected naked DNA and replicating virus DNA (1–5). Alternatively, in vitro ligation (6–8) can be used to generate recombinant vaccinia genomes that can be subsequently recovered as infectious virus with the aid of a helper virus (see Chapters 5 and 6).

In the general in vivo recombination procedure and to facilitate proper insertion of the foreign DNA at a given genome position, viral sequences (often referred to as “flanks”) are included in the transfected DNA. Virus

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recombinants are subsequently isolated from the progeny virus, where only a small percentage (usually 10^{-4} to 10^{-3}) of the total virus are virus recombinants. Thus, the isolation of a vaccinia recombinant implies the selection of low-frequency viruses, a task that is facilitated by the incorporation of genetic markers in the transfected DNA.

Genetic markers used in the vaccinia virus context include genes that can be:

1. Detected by staining (e.g., β -galactosidase and β -glucuronidase) or fluorescence (e.g., GFP; **9–13**).
2. Selected by specific drugs (e.g., neomycin, puromycin, or mycophenolic acid [MPA]; **14–17**).
3. Required for virus spread (e.g., viral genes, A27L or F13L; **18,19**).
4. Required for virus replication in particular cell lines (e.g., host range genes; **20–22**).

One important consideration is the difference between markers intended for the detection of virus recombinants and selectable markers that allow a true selection of the virus recombinants. In both cases, isolation of virus recombinants can be achieved by repeated virus plaquing, but generally selection systems permit a faster and more efficient isolation process.

Most selection systems rely on the use of specific antibiotics or cell lines. One notable exception is the selection system that takes advantage of the nonplaquing phenotype of certain vaccinia deletion mutants. From one such mutant, reintroduction of one particular viral gene results in the restoration of the plaque phenotype. This chapter focuses on the use of one antibiotic resistance system (gpt selection) and one plaque selection system (based on the F13L gene). Alternative methods based on fluorescent protein or β -galactosidase detection are slight variations of the methods described are addressed as **Notes**.

2. Materials

1. Six-well tissue culture plates.
2. 25-cm² and 150-cm² tissue culture flasks.
3. FuGENE 6 transfection reagent (Roche).
4. Complete Eagle's minimum essential medium (EMEM; Gibco BRL) supplemented with 2 mM glutamine, 0.1 μ g/mL penicillin, and 0.1 μ g/mL streptomycin.
5. Cell growth medium: complete EMEM containing 5% fetal bovine serum (FBS).
6. Cell infection medium: complete EMEM containing 2% FBS.
7. Cell lines: CV-1 (ATCC, cat. no. CCL-70) and BSC-1 (ATCC, cat. no. CCL-26) cells.
8. Calcium phosphate transfection buffer (HBS): 0.14 M NaCl, 5 mM KCl, 1 mM Na₂HPO₄·2H₂O, 0.1% dextrose, 20 mM HEPES. Adjust pH precisely to 7.05 by adding 0.5 M NaOH, and filter-sterilize.
9. Water bath sonicator.
10. 2.5 M CaCl₂.

11. Humidified incubator, 37°C, 5% CO₂.
12. Disposable sterile scraper.
13. 5 mL Polypropylene tubes.
14. 5 mL Polystyrene tubes.
15. Complete 2X EMEM medium: 2X EMEM, supplemented with 4 mM glutamine, 0.2 µg/mL penicillin, 0.2 µg/mL streptomycin, and 4% FBS.
16. 2% low-melting point (LMP) agarose in water, autoclaved.
17. 4% X-gal in dimethylformamide.
18. 10 mg/mL neutral red in water.
19. Sterile Pasteur pipets.
20. 10 mg/mL MPA in 0.1 N NaOH.
21. 10 mg/mL xanthine in 0.1 N NaOH.
22. 10 mg/mL hypoxanthine in water.
23. GPT selection medium: complete EMEM containing 1/400 vol of 10 mg/mL MPA, 1/40 vol 10 mg/mL xantine, and 1/670 vol of 10 mg/mL hypoxanthine.
24. 45°C water bath.
25. 5 mg/mL crystal violet staining solution.

3. Methods

F13L selection system is based on virus plaque formation and does not require the use of drugs or antibiotics. The system is based on the observation that vaccinia virus mutants in which most of the F13L coding sequence is deleted are severely impaired in virus transmission, producing tiny virus plaques only after prolonged incubation times (**23,24**). The F13L selection system described here relies on the use of one such mutant (virus vRB12) and a donor plasmid (pRB-21 or derivatives) that restore the complete F13L open-reading frame (ORF) and can simultaneously introduce a foreign gene (**19**). In the plasmid, the F13L coding sequence is flanked by vaccinia virus DNA that directs homologous recombination into the natural F13L locus, placing the foreign gene (under a vaccinia virus promoter) in an intergenic region immediately downstream of the F13L stop codon. Recombinant plaques (normal size plaques) can be distinguished easily from parental plaques (tiny plaques) after a standard 2 d vaccinia-plaque assay.

gpt selection is based on the incorporation in the vaccinia genome of *Escherichia coli gpt* gene and its expression under the control of vaccinia virus promoters. *gpt* expression confers resistance to MPA, an inhibitor of the enzyme inosine monophosphate dehydrogenase (**17,25**). MPA treatment results in depletion of purine nucleotide pools and, consequently, in the inhibition of virus growth. Recombinant viruses expressing *gpt* can be isolated in a selective medium containing MPA and the nucleotide precursors, xanthine and hypoxanthine (see **Note 1**).

3.1. Plasmids

3.1.1. Plasmids for Use by Plaque Selection System

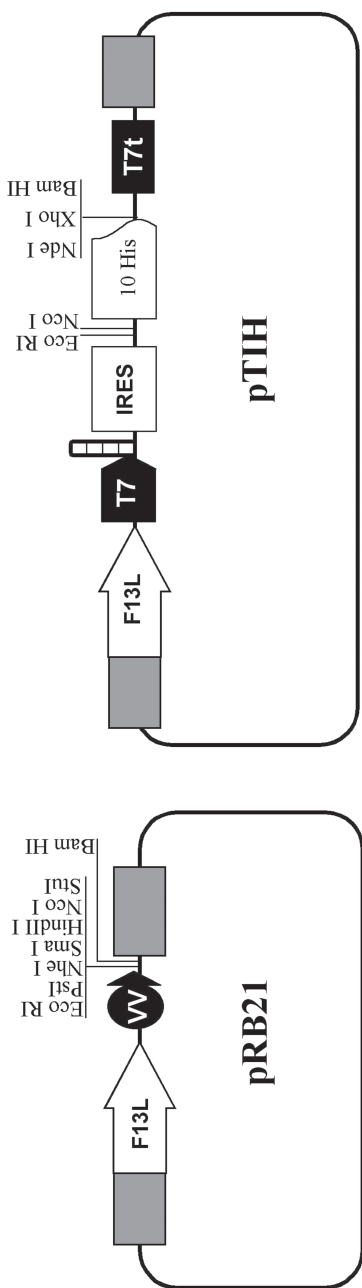
Two different plasmids (**Fig. 1**) have been designed for F13L selection and are used in conjunction with virus vRB12.

1. pRB21 (**19**) is designed to mediate constitutive expression of the foreign gene from a synthetic vaccinia early/late promoter (**26**; see **Fig. 1** and **Note 2**). However, occasionally the expression of the foreign protein interferes with the replication of vaccinia. In such cases, or when the expression of the foreign protein is not desired during the isolation of the recombinant, the donor plasmid pTIH can be used (see **Note 3**).
2. pTIH is designed to place the foreign gene under the control of a T7 promoter (**27**; see **Fig. 1** and **Note 4**). This plasmid was derived from pRB21 and contains the T7 promoter, a sequence to generate a hairpin loop structure at the 5' end of the T7 transcript and an internal ribosomal entry site (IRES) element to facilitate translation initiation. The plasmid contains a N-terminal tag sequence including 10-histidine codons to allow the easy purification of expressed proteins.

3.1.2. Plasmids for Use by gpt Selection System

A number of plasmids have been designed for *gpt* selection (**17,25**). From those, a *gpt* expression cassette, including a vaccinia promoter and the *gpt*-coding sequence, can be easily excised (e.g., see plasmid pTK61-*gpt* in **ref. 17** or plasmid pGEM-*gpt* in **ref. 28**). The *gpt* cassette has been used extensively for insertional mutagenesis of vaccinia virus genes to generate knockout virus mutants. When expression of a foreign gene is desired, plasmids including (in addition to the *gpt* cassette) strong promoters and convenient restriction sites, can be used (see plasmids pTKgpt-F1-3s in **ref. 17**).

Fig. 1. (*opposite page*) Plasmids for F13L selection. Schematic diagram showing relevant features of plasmids pRB21 and pTIH. Relative locations of F13L-coding sequence, vaccinia virus early-late promoter (VV), T7 promoter (T7), T7 terminator (T7t), internal ribosomal entry site (IRES), and unique restriction sites are shown. Vaccinia flanking sequences for recombination in the vaccinia genome are drawn as gray boxes. The lower part shows the sequences downstream of the promoters and the location of restriction sites useful for insertion of foreign genes. TERM, vaccinia virus early transcription termination signal. Note that the polyhistidine tag is positioned at the N-terminus of proteins expressed from open-reading frames (ORF) cloned into the *Nde*I, *Xho*I, or *Bam*HI sites. Foreign genes can be subcloned between pRB21 and pTIH by using restriction sites, *Eco*RI and *Bam*HI.



pRB21

GGAATATAAATAAGGAATTCTGTCAGGCTAGCCCCGGGAAGCTTCATGGAGGCCCTAAATAATAATTTTATGGATCCGGAGAGCT
 EcoRI NheI HindIII StuI BamHI
 PstI SmaI NcoI TERM

pTIH

 IRES

 T7t
 EcoRI NcoI XhoI BamHI
 TGGGAATTCGATATACCATGGGCAT.....CACAGCAGCGGCATATCGAAGTCGTGCATATGCTCGAGGATCCG—
 MetGlyHis (His x 8) HisSerSerGlyGisIleGluGlyArgHisMetLeuGluAspPro...

Fig. 1.

3.2. Infection/Transfection Procedure

To allow recombination between the plasmid and the replicating virus DNA, cells infected with the parental virus are transfected with a plasmid that carries the selective marker. For the F13L selection system, parental virus must be a F13L deletion mutant, such as vRB12 (**19,29**). The nonplaquing phenotype of F13L(−) viruses should be taken into account when growing and titering the virus (*see Note 5*). When using *gpt* selection or β -galactosidase screening, the parental virus can be any normal plaquing virus devoid of those genes.

3.2.1. Transfection with Calcium Phosphate Precipitate

1. About 24 h before infection, split 1:3 a confluent culture of CV-1 cells and seed in a six-well tissue culture plate, in 2 mL cell growth medium per well. Incubate the cells at 37°C in a CO₂ incubator for 20–24 h when the cells should be 60–80% confluent.
2. Prepare virus inoculum (1 mL/well) by diluting a crude virus stock (*see Note 6*) in cell infection medium. The amount of virus should be adjusted to give a multiplicity of infection of 0.05 plaque-forming units cell.
3. Remove cell growth medium from the cultures and immediately add virus inoculum. Place in a CO₂ incubator at 37°C for 1–2 h.
4. About 45 min before transfection, prepare calcium phosphate precipitate as follows. For each transfection, dilute 5 μ g plasmid DNA in 0.5 mL HBS in a 12 \times 75-mm (5 mL) polystyrene tube. Add 25 μ L 2.5 M CaCl₂ droplet by droplet, mixing between each droplet. Leave at room temperature for 20–30 min to allow for a fine DNA–calcium phosphate precipitate to form. The solution should turn opalescent.
5. Aspirate virus inoculum from the CV-1 cell cultures, and add the precipitate on top of the monolayer. Maintain 30 min at room temperature, rocking the plates every 5 min to prevent drying of the monolayers.
6. Add 2 mL per well of cell growth medium. Incubate 4 h in a CO₂ incubator at 37°C.
7. Remove medium from the cultures, and replace with 2 mL fresh cell growth medium. Incubate 72 h in a CO₂ incubator at 37°C.
8. Harvest the infected cells from the well with a disposable rubber scraper or by pipetting repeatedly on top of the monolayer. Collect the cell suspension in a sterile 5-mL polypropylene tube.
9. Release progeny virus from cells by repeated freeze–thawing of the harvested cells by first placing them in dry ice/ethanol bath and then thawing them in a 37°C water bath and vortexing. Repeat the freeze–thaw cycling three times. The cell lysate can be stored at −80°C until plaque isolation is done (*see Subheading 3.3.*).

3.2.2. Transfection with FuGENE

1. Follow **Subheading 3.2.1., steps 1–3.**
2. About 45 min before transfection, prepare transfection mix, in 1.5-mL polypropylene tubes. For each transfection, dilute 6 μ L FuGENE in 94 μ L serum-free EMEM and incubate at room temperature for 5 min. Transfer the EMEM–FuGENE mix to a tube containing 3 μ g plasmid DNA, mix gently by pipeting up and down two or three times. Leave at room temperature for 30 min.
3. Aspirate virus inoculum from the CV-1 cell cultures and wash the cells once with 2 mL serum-free EMEM.
4. Immediately add the DNA–FuGENE solution drop-by-drop on top of the monolayer. Add 2 mL cell infection medium. Incubate 72 h in a CO₂ incubator at 37°C.
5. Then, follow **Subheading 3.2.1., steps 8 and 9.**

3.3. Plaque Isolation

The virus stock obtained at the completion of **Subheading 3.2.** includes mainly parental virus and some recombinant viruses. To isolate the recombinant virus away from the parental virus, individual recombinant virus plaques are picked and characterized. To ensure complete removal of the parental virus, several consecutive rounds of plaque purification are performed. It is advisable to pick and amplify several (e.g., four is a reasonable number) independent plaques in the first plaquing step until an initial characterization is made (*see Subheading 3.5.*). The plaque isolation protocols described here for F13L and *gpt* selection can be used with slight modifications for β -galactosidase (**Note 7**) or fluorescent protein (**Note 8**) detection.

3.3.1. Plaque Isolation: F13L Selection

F13L selection is based solely on virus plaque size and therefore does not require the use of drugs or special cell lines. Any cell line giving good-size wild-type vaccinia plaques, such as BSC-1 cells, can be used to isolate recombinant plaques. It is advisable to perform control transfections with pRB21 and a negative control plasmid (lacking F13L gene) to monitor the presence of recombinant large-virus plaques (*see Fig. 2*).

1. Plate BSC-1 cells in six-well tissue culture plates and incubate until the cells are 90% confluent.
2. Thaw the cell lysate obtained at the completion of **Subheading 3.2.**, remove 0.5 mL, and place in a sterile 5 mL polystyrene tube.
3. Sonicate this in an ice/water bath sonicator for at least three cycles of 15 s until the material in the suspension is dispersed (*see Note 9*).
4. Make 10-fold serial dilutions of the infection/transfection cell lysate in cell infection medium (*see Note 10*).

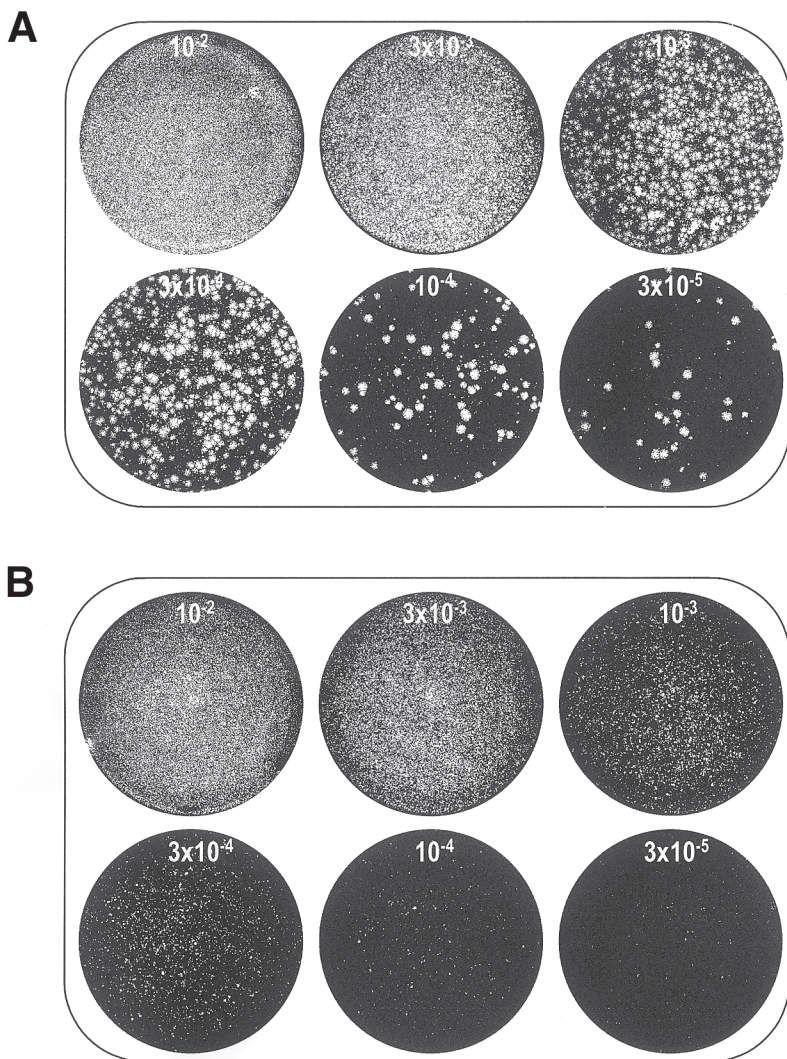


Fig. 2. Plaques of the infection/transfection progeny virus in F13L selection. An example of a first plaquing step after the infection/transfection with virus vRB12 and a plasmid derived from pRB21 (**A**) or a control plasmid not carrying gene F13L (**B**). The dilutions used to infect monolayers of BSC-1 cells in six-well plates are indicated inside each well. Recombinant viruses that have incorporated the F13L gene produce large vaccinia plaques. Note the diffuse cytopathic effect produced by the parental virus in dilutions 10^{-2} and 3×10^{-3} .

5. Aspirate off growth medium from the BSC-1 cell monolayers and add 1 mL of the corresponding dilution per well. (Use at least dilutions 10^{-2} , 10^{-3} , and 10^{-4} .) Incubate 1–2 h at 37°C.
6. Melt sterile 2% LMP agarose in water by heating in a microwave oven and cool in a 45°C water bath. Warm complete 2X EMEM medium at 37°C.
7. Immediately before use, prepare EMEM–agarose overlay by mixing equal volumes of complete 2X EMEM medium and 2% LMP agarose solution.
8. Remove virus inoculum from each well and add 3 mL EMEM–agarose overlay medium. Allow overlay to solidify at room temperature (*see Note 11*). Then, place the plates in the 37°C incubator for 48 h to allow for vaccinia plaques to develop.
9. Stain the monolayer by overlaying the EMEM agarose with the addition of 2 mL EMEM–agarose containing neutral red. This overlay is prepared by mixing equal volumes of complete 2X EMEM containing 1/100 vol of 10 mg/mL neutral red and 2% LMP agarose (agarose has been melted and cooled at 45°C). Add 2 mL of this cell-staining overlay to each well and allow to solidify at room temperature. Then, place plates in the 37°C incubator until vaccinia plaques are visible (5–16 h).
10. When plaques are clearly visible, pick well-separated plaques by plunging a sterile Pasteur pipet through the agarose medium all the way to the plastic. Rock the pipet tip slightly to scrape and detach cells in the plaque. Gently aspirate (*see Note 12*) the agarose plug and transfer to a microcentrifuge tube containing 0.5 mL cell growth medium. Freeze–thaw three times and sonicate.
11. Repeat **steps 1–10** three to five times, each time using the material from the last plaque selected.

3.3.2. Plaque Selection: *gpt* Selection

This selection system is based on plaque isolation in the presence of MPA, an inhibitor of purine metabolism (**17,25**). Virus recombinants incorporating and expressing the *E. coli* xanthine–guanine phosphoribosyl transferase gene (*gpt*), but not normal *gpt*([−]) virus, can form plaques in medium containing MPA and the nucleotide precursors xanthine and hypoxanthine (*see Fig. 3*).

1. Seed BSC-1 cells in six-well tissue culture plates and incubate until the cells are approx 90% confluent.
2. Twelve to twenty-four hours before infection, prepare GPT selection medium. Remove cell growth medium from wells and replace with 2 mL/well of freshly prepared GPT selection medium.
3. Thaw the cell lysate obtained at the completion of **Subheading 3.2.**, remove 0.5 mL, and place in a sterile 5-mL polystyrene tube.
4. Sonicate in an ice/water bath sonicator for at least three cycles of 15 s until the material in the suspension is dispersed (*see Note 9*).
5. Make 10-fold serial dilutions of the infection/transfection cell lysate in cell infection medium. Aspirate GPT selection medium from the BSC-1 cell mono-

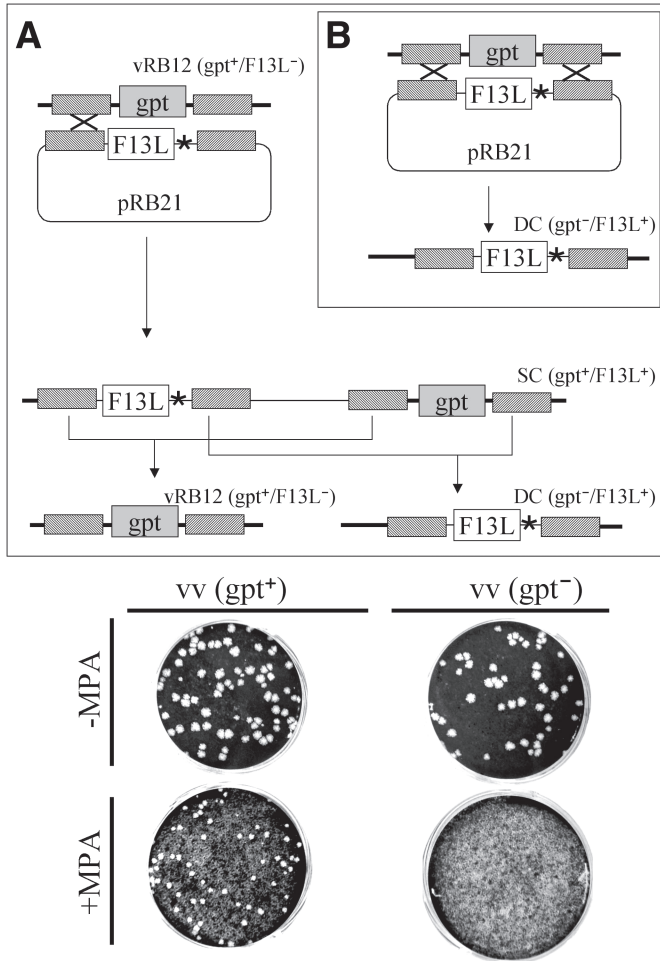


Fig. 3. *gpt* screening assay. The recombination of the plasmid with the vaccinia genome may occur at only one flanking sequence (A) or at both flanking sequences (B). To distinguish between the unstable simple crossover (SC) products (*gpt*⁺, F13L⁺) and the stable double crossover (DC) products (*gpt*⁻, F13L⁺), a *gpt* assay can be done on the growth of plaque-purified recombinant viruses. The lower panel shows the virus plaques obtained with *gpt*⁻ and *gpt*⁺ viruses in nonselective (-MPA) or selective (+MPA) medium. (The position of the promoter/foreign gene DNA sequence in A and B is indicated by an asterisk.)

layers and add 1 mL of the corresponding dilution per well. (Use at least dilutions 10⁻², 10⁻³, and 10⁻⁴.) Incubate 1–2 h at 37°C to allow virus adsorption.

- Before the end of the adsorption period, melt the 2% LMP agarose in water by heating in a microwave oven and cool in a 45°C water bath. Supplement com-

plete 2X EMEM medium with 1/200 vol of 10 mg/mL MPA, 1/20 vol 10 mg/mL xantine, and 1/335 vol of 10 mg/mL hypoxantine, and warm at 37°C.

7. Immediately before use, prepare EMEM–agarose overlay by mixing equal volumes of the solutions prepared in **step 6** (selective EMEM–agarose overlay medium).
8. Remove virus inoculum from each well and add 3 mL selective EMEM–agarose overlay medium. Allow to solidify at room temperature (*see Note 11*). Place the plates in the 37°C incubator for 48 h to allow for vaccinia plaques to develop.
9. Stain the monolayer and pick plaques as described in **Subheading 3.3.1., steps 9–11**.

3.4. Virus Amplification

After the three to five consecutive rounds of plaque purification, virus recombinants from the last plaques are amplified by infection of monolayer cells to make a first-virus crude stock.

1. Seed BSC-1 cells in a six-well tissue culture plate and incubate in a CO₂ incubator until the cells are confluent.
2. Infect one well with 250 µL of the material from a plaque diluted to 1 mL with cell infection medium. Place the plates in a CO₂ incubator for 2 h at 37°C.
3. After the 2-h adsorption period, remove virus inoculum and add 2 mL fresh cell infection medium. Place at 37°C in a CO₂ incubator for 48–72 h (i.e., until cytopathic effect is complete).
4. Detach the cells from the plastic with a disposable rubber scraper or by repeatedly pipeting on top of the monolayer. Transfer the cell suspension to a tube. Freeze–thaw three times and sonicate (*see Note 9*).
5. Use half of the cell lysate, sonicated and diluted to 4 mL in cell infection medium to infect a confluent BSC-1 monolayer culture in a 75-cm² flask. After 2-h adsorption in a CO₂ incubator at 37°C, remove virus inoculum, add 12 mL cell infection medium, and place in the incubator at 37°C for 48–72 h (i.e., until cytopathic effect is complete).
6. Detach infected cells from the flask with a disposable scraper and transfer to a 15-mL centrifuge tube. Centrifuge 5 min at 1800g, discard supernatant, and resuspend the cells in 1 mL complete EMEM 2% FBS. Titrate virus stock (*see Chapter 8*).

3.5. Initial Screening of Resulting Recombinant Viruses by Growth Under *gpt* Selection

A first step in the characterization of the viruses isolated by F13L selection and amplified is to check whether they are “single” or “double” recombinants. Single recombinants are highly unstable as they contain direct repeats, producing by recombination both the parental virus and the desired recombinant virus (*see Fig. 3*). Because parental vRB12 virus contains a *gpt* cassette that replaces most of the F13L gene, single recombinants maintain the *gpt*(+) phenotype.

Thus, the presence of *gpt*(⁺) viruses in the amplified virus stock is an indication that single recombinants were picked in the plaque isolation process. Conversely, complete absence of *gpt*(⁺) viruses in the stock indicate that a desired recombinant has been successfully isolated. Thus, virus plaquing under *gpt*-selective conditions provides a rapid method in determining if the stock is contaminated with any single recombinants (*see* **Note 13**).

1. Seed BSC-1 cells in cell growth medium in six-well tissue culture plates and incubate until the monolayers reach a 80–90% confluency.
2. Twelve to twenty hours before plaquing, half of the wells are preincubated in 2 mL GPT selection medium. When cells are pretreated with GPT selection medium, change the medium of the remaining wells (control wells) to 2 mL cell infection medium.
3. Thaw the virus stock (*see* **Note 14**) and make serial dilutions in 2 mL cell infection medium.
4. Remove medium and infect monolayers with the virus dilutions using 1 mL/well. Infect one well with each dilution that had been pretreated with GPT selection medium and one control well. Incubate at 37°C for 1 h.
5. Aspirate virus inocula and add 2 mL GPT selective medium to the pretreated wells and 2 mL cell infection medium to the control wells. Incubate 48 h in the 37°C incubator.
6. Stain the monolayers by adding 0.5 mL/well of 5 mg/mL crystal violet solution. Mix with the medium by moving the plate carefully. Leave at room temperature for at least 10 min.
7. Aspirate stained medium from the wells. Monolayers and virus plaques should be clearly visible. Let the monolayers dry at room temperature. Confirm that there are no plaques in the wells with GPT selective medium on them.

4. Notes

1. Recombinant viruses that have *gpt* inserted into the viral genome can be used as parental viruses to introduce other mutations into the virus using reverse *gpt* selection (**30**).
2. Plasmid pRB21 (**19**) is designed for expression of a foreign gene from a synthetic early–late vaccinia promoter (**26**). Many proteins can be expressed to high levels with the resulting recombinants. Note that if the foreign sequence contains a TTTTNT (an early transcriptional stop signal; *see* Chapter 11) within the ORF, truncated proteins may be expressed at early times during infection.
3. It is likely that before constructing a virus recombinant, the toxicity of the foreign protein may not be predicted. In those cases, it is advisable to make constructs in both plasmids pRB21 and pTIH, and transfect them in parallel. The insert can usually be transported between those plasmids by using the *EcoRI*, *NcoI*, or *BamHI* sites present in both plasmids. After the infection/transfection, the number of virus plaques obtained with both constructs is a good indication of the toxicity of the protein in the vaccinia virus context.

4. pTIH allows one to carry out the isolation of the virus recombinant in the absence of expression of the foreign gene. Eventually, expression is induced by providing the T7 RNA polymerase *in trans* (27). This can be achieved by infecting cell lines that stably express T7 RNA polymerase or by coinfection with vaccinia vTF7-3 (31), a recombinant in which the T7 RNA polymerase gene is placed under the control of a vaccinia promoter (*see* Chapter 4).
5. vRB12 growth and titration. Because vRB12 is not readily exported to the medium or transmitted between cells, efficient amplification of the virus requires mechanical release of the virus replicated within cells and consecutive rounds of amplification. For amplification, it is advisable to use moi more than 1 pfu/cell to ensure an efficient infection of the monolayer (and no requirement for cell-to-cell virus spread in the culture). It is difficult to accurately determine the titers of VRB12 virus stocks, as the virus does not form virus plaques after the standard vaccinia 2-d plaquing assay. However, virus titers can be estimated either by inspection of the cytopathic effect caused by different dilutions of the virus stocks or, preferably, by counting the number of small plaques that form after a 5–7-d plaquing assay.
6. For F13L selection, use virus vRB12 or a suitable F13L deletion mutant as parental virus. For *gpt* selection, use any vaccinia virus that does not contain the *gpt* gene as the parental virus.
7. Isolation of β -galactosidase-expressing viruses involves a normal plaque isolation protocol (e.g., described in **Subheading 3.3.**), in which the chromogenic β -galactosidase substrate X-gal is added in the overlay medium. Thus, in **Subheading 3.3., step 9**, supplement the EMEM 2X medium with 1/120 vol of a solution of 4% (w/v) X-gal in dimethylformamide. After a few hours, blue color for the recombinant plaques will start to develop. Note that β -galactosidase detection is not a true selection, and usually parental virus is usually carried along with the recombinant for at least two or three plaquing rounds. Thus, in this case, a minimum of five to six rounds of plaque selection (i.e., until all the plaques appear blue) is recommended.
8. Detection of recombinant plaques expressing fluorescent proteins is optimally carried out using an inverted fluorescence microscope. Direct inspection of the plaques enables marking the position of fluorescence-positive plaques that can be subsequently picked. We have routinely used a standard FITC filter set (excitation: 465–495 nm; dichroic mirror: 505; emission: 515–555 nm) for GFP visualization and a standard Rhodamine filter set (excitation: 515–560 nm; dichroic mirror: 575 nm; emission: BA590) for dsRed visualization.
9. Sonication seems to work best when carried out on a small volume of lysate in rigid-wall tubes. We find that sonication of the virus in 5-mL or 15-mL tubes works better than when done in microcentrifuge tubes. Care must be taken not to overheat the specimen during sonication because high temperature inactivates the virus.
10. In the F13L system, the first plaquing of the progeny virus from the infection/transfection is a crucial step. Usually, large plaques are clearly visible in the

monolayers, but keep in mind that many more cells infected by the parental virus are present and replicate in the cultures. If the amount of parental virus is high, a diffuse cytopathic effect appears, which makes it difficult to see large-sized plaques. Consequently, making two- or threefold dilutions is recommended for the first plaquing cycle (see **Fig. 2**).

11. Optionally, place plates at 4°C for 10–15 min to ensure proper solidification of the agarose overlay.
12. When aspirating the agarose plug into the Pasteur pipet, it is best to try to aspirate the agarose plug only as high as the pipet tip rather than into the body of the pipet. This will make the transfer into the tube easier and minimize potential loss of the plaque.
13. This initial type of screen is only useful to perform when the parental virus contains the *gpt* gene. For recombinant viruses that have been isolated by inserting the *gpt* gene into the virus (**Subheading 3.3.2.**), this type of screening will not work. Because looking for a difference in the number of plaques grown in the presence or absence of GPT selection medium will not identify if parental wild-type virus is contaminating the recombinant stock, as titering under *gpt* selection typically results in a lower plaque count than when titering without selective medium.
14. This initial check can be done using a small amounts of virus that are from earlier steps of the amplification of the purified plaque (e.g., **Subheading 3.4., step 4**).

References

1. Moss, B. and Flexner, C. (1987) Vaccinia virus expression vectors. *Annu. Rev. Immunol.* **5**, 305–324.
2. Moss, B. and Flexner, C. (1989) Vaccinia virus expression vectors. *Ann. NY Acad. Sci.* **569**, 86–103.
3. Moss, B. (1992) Vaccinia virus vectors. *Biotechnology* **20**, 345–362.
4. Moss, B. (1996) Genetically engineered poxviruses for recombinant gene expression, vaccination, and safety. *Proc. Natl. Acad. Sci. USA* **93**, 11341–11348.
5. Earl, P. and Moss, B. Expression of proteins in mammalian cells using vaccinia viral vectors, in *Current Protocols in Molecular Biology* (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., Struhl, K., eds.), Wiley-Interscience, New York, 1991.
6. Scheifflinger, F., Dorner, F., and Falkner, F. G. (1992) Construction of chimeric vaccinia viruses by molecular cloning and packaging. *Proc. Natl. Acad. Sci. USA* **89**, 9977–9981.
7. Merchlinsky, M. and Moss, B. (1992) Introduction of foreign DNA into the vaccinia virus genome by in vitro ligation: recombination-independent selectable cloning vectors. *Virology* **190**, 522–526.
8. Merchlinsky, M., Eckert, D., Smith, E., and Zauderer, M. (1997) Construction and characterization of vaccinia direct ligation vectors. *Virology* **238**, 444–451.
9. Chakrabarti, S., Brechling, K., and Moss, B. (1985) Vaccinia virus expression vector: coexpression of beta-galactosidase provides visual screening of recombinant virus plaques. *Mol. Cell Biol.* **5**, 3403–3409.

10. Panicali, D., Grzelecki, A., and Huang, C. (1986) Vaccinia virus vectors utilizing the beta-galactosidase assay for rapid selection of recombinant viruses and measurement of gene expression. *Gene* **47**, 193–199.
11. Carroll, M. W. and Moss, B. (1995) E. coli beta-glucuronidase (GUS) as a marker for recombinant vaccinia viruses. *Biotechniques* **19**, 352–354, 356.
12. Dominguez, J., Lorenzo, M. M., and Blasco, R. (1998) Green fluorescent protein expressed by a recombinant vaccinia virus permits early detection of infected cells by flow cytometry. *J. Immunol. Methods* **220**, 115–121.
13. Lorenzo, M. M. and Blasco, R. (1998) PCR-based method for the introduction of mutations in genes cloned and expressed in vaccinia virus. *Biotechniques* **24**, 308–313.
14. Franke, C. A., Rice, C. M., Strauss, J. H., and Hruby, D. E. (1985) Neomycin resistance as a dominant selectable marker for selection and isolation of vaccinia virus recombinants. *Mol. Cell Biol.* **5**, 1918–1924.
15. Katz, J. B. and Middle, L. A. (1990) Evaluation of thymidine kinase and neomycin phosphotransferase II positive selection systems for recovery of genetically atypical and recombinant DNA vaccine viruses. *Biologicals* **18**, 301–304.
16. Sanchez-Puig, J. M. and Blasco, R. (2000) Puromycin resistance (pac) gene as a selectable marker in vaccinia virus. *Gene* **257**, 57–65.
17. Falkner, F. G. and Moss, B. (1988) Escherichia coli gpt gene provides dominant selection for vaccinia virus open reading frame expression vectors. *J. Virol.* **62**, 1849–1854.
18. Rodriguez, J. F. and Esteban, M. (1989) Plaque size phenotype as a selectable marker to generate vaccinia virus recombinants. *J. Virol.* **63**, 997–1001.
19. Blasco, R. and Moss, B. (1995) Selection of recombinant vaccinia viruses on the basis of plaque formation. *Gene* **158**, 157–162.
20. Staib, C., Drexler, I., Ohlmann, M., Wintersperger, S., Erfle, V., and Sutter, G. (2000) Transient host range selection for genetic engineering of modified vaccinia virus Ankara. *Biotechniques* **28**, 1137–1142, 1144–1146, 1148.
21. Perkus, M. E., Limbach, K., and Paoletti, E. (1989) Cloning and expression of foreign genes in vaccinia virus, using a host range selection system. *J. Virol.* **63**, 3829–3836.
22. Smith, K. A., Stallard, V., Roos, J. M., Hart, C., Cormier, N., Cohen, L. K., et al. (1993) Host range selection of vaccinia recombinants containing insertions of foreign genes into non-coding sequences. *Vaccine* **11**(1), 43–53.
23. Blasco, R. and Moss, B. (1991) Extracellular vaccinia virus formation and cell-to-cell virus transmission are prevented by deletion of the gene encoding the 37,000-dalton outer envelope protein. *J. Virol.* **65**, 5910–5920.
24. Blasco, R. and Moss, B. (1992) Role of cell-associated enveloped vaccinia virus in cell-to-cell spread. *J. Virol.* **66**, 4170–4179.
25. Boyle, D. B. and Coupar, B. E. H. (1988) A dominant selectable marker for the construction of recombinant poxviruses. *Gene* **65**, 123–128.
26. Chakrabarti, S., Sisler, J. R., and Moss, B. (1997) Compact, synthetic, vaccinia virus early/late promoter for protein expression. *Biotechniques* **23**, 1094–1097.

27. Galindo, I., Lorenzo, M. M., and Blasco, R. (2001) Set of vectors for the expression of histidine-tagged proteins in vaccinia virus recombinants. *Biotechniques* **30**, 524–526, 528–529.
28. Blasco, R., Cole, N. B., and Moss, B. (1991) Sequence analysis, expression, and deletion of a vaccinia virus gene encoding a homolog of profilin, a eukaryotic actin-binding protein. *J. Virol.* **65**, 4598–4608.
29. Blasco, R. and Moss, B. (1992) Role of cell-associated enveloped vaccinia virus in cell-to-cell virus spread. *J. Virol.* **66**, 4170–4179.
30. Isaacs, S. N., Kotwal, G. J., and Moss, B. (1990) Reverse guanine phosphoribosyl-transferase selection of recombinant vaccinia viruses. *Virology* **178**, 626–630.
31. Fuerst, T. R., Niles, E. G., Studier, F. W., and Moss, B. (1986) Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. *Proc. Natl. Acad. Sci. USA* **83**, 8122–8126.

Vaccinia Virus and Poxvirology

Methods and Protocols

Isaacs, S.N. (Ed.)

2004, XX, 396 p., Hardcover

ISBN: 978-1-58829-229-2

A product of Humana Press