PROTOCOL

Recombinant Vaccinia Viruses

Design, Generation, and Isolation†

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Abstract

The technologies of recombinant gene expression have greatly enhanced the structural and functional analyses of genetic elements and proteins. Vaccinia virus, a large double-stranded DNA virus and the prototypic and best characterized member of the poxvirus family, has been an instrumental tool among these technologies and the recombinant vaccinia virus system has been widely employed to express genes from eukaryotic, prokaryotic, and viral origins. Vaccinia virus is also the prototype live viral vaccine and serves as the basis for well established viral vectors which have been successfully evaluated as human and animal vaccines for infectious diseases and as anticancer vaccines in a variety of animal model systems. Vaccinia virus technology has also been instrumental in a number of unique applications, from the discovery of new viral receptors to the synthesis and assembly of other viruses in culture. Here we provide a simple and detailed outline of the processes involved in the generation of a typical recombinant vaccinia virus, along with an up to date review of relevant literature.

Index Entries: Vaccinia virus; biotechnology/methods; proteins/biosynthesis; genetic vectors; recombinant proteins; gene expression; recombination; selection/genetics; transfection.

1. Introduction

The structural and functional analyses of proteins have benefited enormously from the use of technologies of recombinant gene expression. The recombinant vaccinia virus system has been widely employed to express genes from eukaryotic, prokaryotic, and viral origins ***(1–11)*** and several detailed protocols for the generation, identification, isolation, and characterization of recombinant vaccinia viruses have been published ***(12–14)***.

Vaccinia virus, a large double-stranded DNA virus, is the prototypic and best characterized member of the poxvirus family. Replication and gene expression occur in the cytoplasm of the infected host cell ***(15,16)***. The expression of vaccinia viral genes occurs in succession through the regulated transcription of early, intermediate, and late classes of genes, as dictated by viral promoter structures ***(17)***. Since the first description and use of the recombinant vaccinia virus expression system in the early 1980s ***(18,19)***, it has been modified, improved, and extensively used. Foreign gene expression by recombinant vaccinia viruses offers several advantages:

1. Proteins are processed and modified correctly.
2. Proteins are properly transported and localized in the infected cell.
3. Uniform protein production is achieved within a target cell population using a high multiplicity of infection (MOI).
4. The extremely broad host range of vaccinia virus allows a wide array of primary and transformed tissue culture cell lines to be utilized.

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5. Foreign gene expression can be achieved with high efficiency in cells that are refractory to nucleic acid transfection procedures such as primary macrophage cultures ***(20).***
6. A variety of natural and synthetic vaccinia virus promoters, as well as hybrid systems using the bacteriophage T7 ***(21–23)***, T3 ***(24)***, and SP6 ***(25)*** promoters, and repression via the *Escherichia coli lac* repressor/operator ***(26–29)*** permit varying levels and control of gene expression.
7. The problems and limitations associated with expression in permanently transformed cell lines (e.g., production of cytotoxic proteins) are avoided due to the transient nature of the vaccinia virus system.
8. The cytoplasmic localization of transcription bypasses requirements for regulated export of unspliced mRNAs out of the nucleus [e.g., for structural proteins of primate lentiviruses ***(30)***]. However, since messenger RNAs (mRNAs) are not spliced in the vaccinia virus system ***(31)***, open reading frames must be continuous.

Vaccinia virus is also the prototype live viral vaccine and serves as the basis for well-established viral vectors that have been successfully evaluated as human and animal vaccines for infectious diseases and as anticancer vaccines in a variety of animal model systems ***(32,33)***. However, vaccinia virus is infectious for humans and its imperfect record of safety as a smallpox vaccine has been a concern for its use as vector in clinical applications. More recently, the development of highly attenuated vaccinia viral vectors was attained through the development of recombinant viruses from modified vaccinia virus Ankara (MVA), a strain with established clinical safety ***(34)***. MVA was generated by long-term serial passage in avian cells and it is characterized by its avirulence and severe deficiency to replicate in cells of mammalian origin ***(35–37)***. Another highly attenuated vaccinia strain is NYVAC, which was constructed by multiple gene deletions affecting host range and pathogenesis ***(38)***. Importantly, these attenuated recombinant vaccinia viruses have been found to be immunogenic and protective against disease when used as candidate recombinant vaccines in animal models for viral or parasitic infections ***(39–49)***.

1.1. The Mechanism of Recombinant Vaccinia Virus Generation

The original and still most widely used method for the generation of recombinant vaccinia viruses relies on homologous recombination in vivo ***(18,19)***. The general scheme for incorporation of foreign coding sequences into the virus genome by homologous recombination is diagrammed in **Fig. 1**. First, the gene of interest is cloned into a plasmid transfer vector that contains the following elements: (1) a vaccinia virus promoter; (2) a multiple cloning site adjacent to the promoter; (3) flanking sequences derived from a nonessential site within the vaccinia virus genome; and (4) the necessary elements for replication and selection in bacteria. In addition, screening and/or selection markers may be included to facilitate identification of recombinant virus. A list of commonly used transfer vectors is shown in **Table 1**. Second, tissue culture cells are infected with a parental strain of vaccinia virus, such as Western Reserve (WR), and transfected with the transfer vector containing the gene of interest. Homologous recombination between the vaccinia virus DNA and the transfer vector results in incorporation of the foreign gene into the viral genome. This recombination process yields approx 1 recombinant virion in 1000 progeny. Replication of the recombinant genome continues and maturation of virions occurs. Third, the desired recombinant vaccinia virus is plaque purified by several rounds of selection and/or screening. Finally, high titer recombinant virus stocks are prepared from infected cell lysates. Purification of vaccinia virus can be performed if the presence of host cell proteins is undesirable or if very high titers of virus are required [1–5 × 10^{10} plaque-forming-units (pfu)/mL].

In addition to generation of recombinant viruses by in vivo homologous recombination with cloned genes of interest, a protocol for the insertion of linear polymerase chain reaction (PCR) generated constructs has been developed that essentially eliminates having to clone the

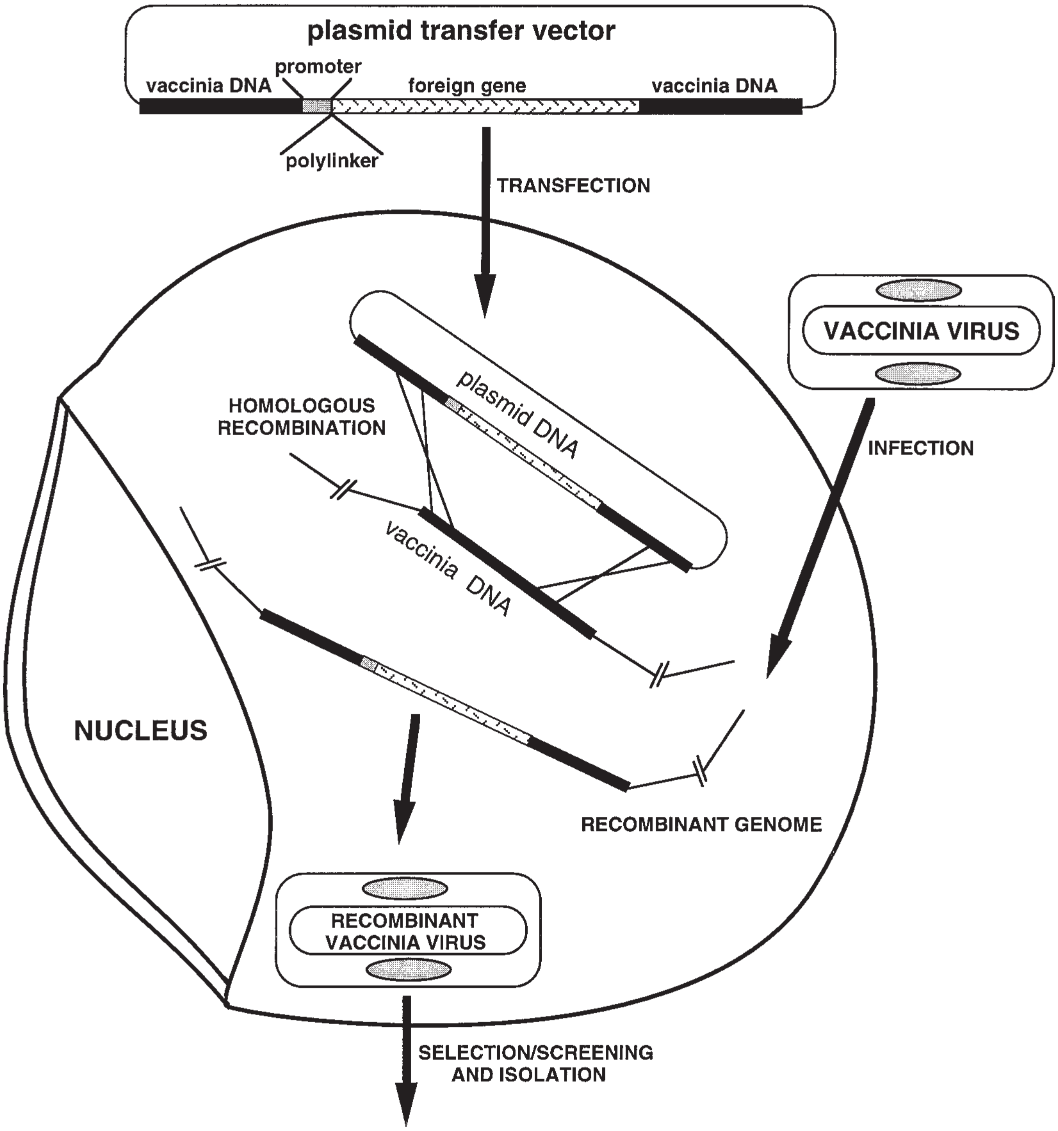

Fig. 1. The generation of recombinant vaccinia viruses by homologous recombination. Cells are infected with vaccinia virus and transfected with a plasmid transfer vector that contains a foreign gene driven by a viral promoter and flanked by vaccinia virus DNA segments. Homologous recombination between the vaccinia virus sequences in the transfected vector DNA and the viral genome occurs during the replication cycle of virus. The resulting DNA genome is packaged to form progeny recombinant vaccinia virus (diagram not drawn to scale).

gene of interest into a transfer vector ***(50)***. Furthermore, protocols for direct in vitro ligation of genomic vaccinia virus DNA "arms" with foreign DNA have been described ***(51,52)*** that allow for viable recombinant viruses to be recovered by rescue with a conditionally lethal vaccinia virus or host-range restricted fowlpox virus. These methods circumvent a need for homologous recombination and incorporation of very large DNA segments (up to 26,000 bp) can be achieved. More recently, these protocols have been improved through the incorporation of pairs of unique restriction enzyme sites into the vaccinia virus genome which allow forced, direct ligation with fixed orientations of foreign DNA segments and eliminates the production of contaminating wild-

Table 1
Vaccinia Virus Transfer Vectors[a]

Selection/Screening	Vector	Promoter[b]	Flanking[c] vaccinia DNA	Reference
TK	pGS20	P7.5 (E/L)	TK	***(97)***
	pSC59	Synthetic (E/L)	TK	***(56)***
TK and β-gal	PMJ601	Synthetic (L)	TK	***(55)***
	pSC65	Synthetic (E/L)	TK	***(56)***
	pSC11[d]	P7.5 (E/L)	TK	***(76)***
β-gal	pCF11	P7.5 (E/L)	*Hind*III C	***(98)***
Ecogpt and/or TK	PTKgptF1s[d]	P11 (L)	TK	***(78)***
	pMC1107	P7.5 (E/L)	TK	***(80)***

[a] Representative plasmid transfer vectors utilizing the types of selection/screening protocols outlined in the chapter are shown. The table in not intended to be exhaustive. A more complete list can be found in reference ***13***.

[b] L, late; E/L, early and late.

[c] Vaccinia virus genome region used for directing homologous recombination.

[d] Represented in **Fig. 2.**

type genomes after religation of viral "arms" ***(53)***. Finally, a combination of the oriented forced ligation method with PCR-generated constructs has provided a novel approach for efficient insertion and expression of genes or even libraries of cDNAs without further cloning steps or need of selectable markers ***(54)***.

1.2. Choice of Transfer Vector and Promoter Considerations

There are numerous combinations of promoter and selection systems. The type of promoter employed dictates both the level and time of expression. Quantitative analysis of the expression of vaccinia virus genes has revealed that early promoters express genes from 0.5 h to a peak at 1.5 h postinfection, intermediate promoters from approx 1.5 h to a peak at 2 h, and late promoters from approx 3 h onward ***(17)***. Constitutive or compound promoters are those that contain both early and late transcriptional elements. Factors that influence the choice of promoter system come from assessing the desired use of the recombinant vaccinia virus, or from the known properties of the gene product of interest. For example, for large-scale protein production a strong vaccinia virus promoter such as the synthetic late ***(55)*** or early/late ***(56)*** promoter or the hybrid vaccinia/T7 polymerase system ***(22,29)*** should be used ***(57,58)***; whether the gene product of interest is secreted or nonsecreted may also need to be addressed to establish conditions for optimal protein production ***(59)***; for induction of class I restricted cytotoxic T cell response in vivo, a natural early or tandem early/late promoter is recommended ***(5)***; for production of a potentially cytotoxic protein use of the *E. coli* lac repressor/operator system ***(29,60)*** or the hybrid vaccinia/T7 system allows for initiation of gene expression when appropriate. When early gene expression is important, the coding sequence should be scanned for the presence of the sequence TTTTTNT. This sequence signals early transcriptional termination in vaccinia virus ***(61)*** and should be changed without altering the amino acid sequence ***(62)***. Finally, if a specialized cell type is to be used, such as primary cell cultures, it may prove useful to characterize that cell type for its ability to support vaccinia virus infection, replication, and gene expression by different classes of promoters ***(63,64)***. This can be achieved in a straightforward manner by use of an available reporter gene (e.g., *E. coli lacZ*) linked to different promoters. In addition, Chinese hamster ovary cells, which are a popular choice for recombinant gene expression for protein production yet normally nonpermissive for vaccinia virus, can now be utilized through the incorporation of the cowpox virus host range gene ***(65)***.

Aside from the aforementioned use as a technique for recombinant protein production and as a live viral vaccine, recombinant vaccinia virus technology has been instrumental in a number of unique applications. For example, the vaccinia-based T7 expression system was critical in the characterization of human immunodeficiency virus type-1 (HIV-1) viral envelope glycoprotein functional requirements for membrane fusion ***(66,67)***, and later in the identification of the first coreceptor ***(68)***. Also, the rescue and production of several infectious RNA viruses using the vaccinia-based T7 expression system has been reported ***(69–75)***.

1.3. Selection and Screening of Recombinant Vaccinia Viruses

One of the most widely used types of transfer vector utilizes recombination into the nonessential thymidine kinase (*tk*) gene of vaccinia. An example of such a vector is pSC11 ***(76)***, shown in **Fig. 2**, panel A. Not only is the *tk* gene nonessential, but disruption of this function provides a means of selecting recombinant viruses with a tk^- phenotype by growth in the presence of the thymidine analog 5-bromodeoxyuridine (BrdU) ***(18)***. Using spontaneous tk^- vaccinia viruses, the first foreign gene to be introduced and expressed in vaccinia virus was the herpes simplex virus *tk* gene ***(18,19)***. Incorporation of a functional *tk* gene into the transfer vector allows selection of recombinant vaccinia viruses with a tk^+ phenotype when using a tk^- parental virus ***(77)***. Another widely used selection mechanism employs the incorporation of the *E. coli* xanthine-guanine phosphoribosyl transferase (XGPRT) gene (*Ecogpt*) into the transfer vector ***(78–80)***. An example is pTKgptF1s, shown in **Fig. 2**, panel B. Mycophenolic acid (MPA), an inhibitor of purine metabolism, blocks replication of vaccinia virus. Expression of *Ecogpt* by vaccinia virus and inclusion of xanthine and hypoxanthine in the growth medium rescues the virus from this blockage. Thus, plasmid transfer vectors which include the *Ecogpt* gene controlled by a vaccinia virus promoter in the recombination cassette will yield recombinant vaccinia viruses expressing both the *Ecogpt* gene and the gene of interest. The use of *Ecogpt* is advantageous for several reasons:

1. Selection of recombinant vaccinia viruses is not restricted to a tk^- cell line
2. Homologous recombination can be directed to any nonessential site in the vaccinia virus genome.
3. Spontaneous MPA resistant mutations do not occur so only *Ecogpt* expressing recombinant viruses will replicate and form a plaque.
4. MPA is nonmutagenic.

In addition, once incorporated into the vaccinia virus genome, the *Ecogpt* gene can be removed using a reverse selection mechanism ***(81)***. The drug 6-thioguanine (6-TG) is toxic to mammalian cells which express *Ecogpt* or hypoxanthine-guanine phosphoribosyl transferase (HGPRT), the mammalian homolog of *Ecogpt*. Thus, HGPRT negative cells are used to select recombinant vaccinia viruses that have undergone homologous recombination to remove the *Ecogpt* gene and replace it with another gene. The ability to select for or against *Ecogpt* expression provides a technique for introducing multiple genes into a recombinant virus through successive rounds of insertion and removal of an *Ecogpt* cassette. A potential disadvantage of this method is that viruses resulting from a single crossover event (containing both the foreign gene and *Ecogpt*) can be stable. If such a virus is isolated, it may later undergo recombination to yield a mixed population containing parental and recombinant viruses. A modification of this method, known as transient dominant selection, provides a means of introducing foreign DNA followed by removal of the *Ecogpt* selection marker ***(82)***. In this method the *Ecogpt* gene is located outside of the vaccinia virus DNA segments in the plasmid transfer vector. Thus, MPA resistant recombinant viruses acquire the *Ecogpt* gene through a single recombination event in which the entire plasmid is incorporated into the virus genome. This arrangement is unstable and the *Ecogpt* gene is readily lost when selection is removed. The transient dominant selection method simplifies the technique for introducing multiple genes in succession, and is advantageous

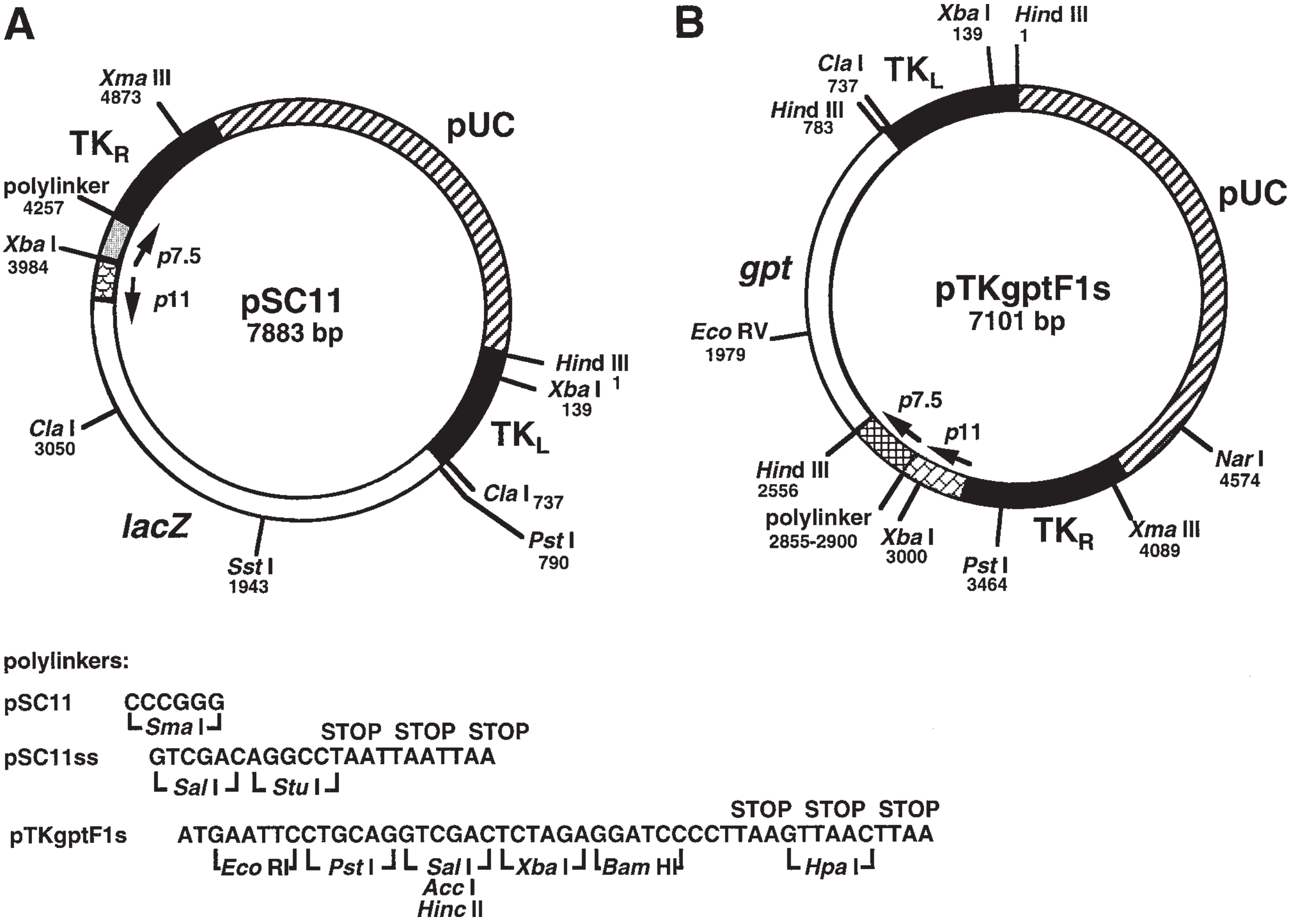

Fig. 2. Examples of plasmid transfer vectors. (**A**) pSC11 ***(76)*** and pSC11ss ***(62)*** provide for both *tk*$^-$ selection and beta-galactosidase screening. The late vaccinia virus promoter *p*11 drives the *E. coli lacZ* gene. Genes can be inserted at the polylinker site and are controlled by the compound early/late vaccinia virus promoter *p*7.5. (B) pTKgptF1s ***(78)*** provides for *tk*$^-$ and/or XGPRT selection. The compound early/late vaccinia virus promoter *p*7.5 drives the expression of the *Ecogpt* gene. The polylinker provides an ATG start and expression is driven by the late vaccinia virus promoter *p*11. Additional variants, pTKgptF2s and pTKgptF3s, contain one or two additional G residues, respectively, following the ATG to allow all three codon phasing possibilities. The expression cassette in both of these plasmids is flanked by segments of the vaccinia virus *tk* gene to direct the insertion into the vaccinia virus genome. Nucleotide numbers for both these plasmids have been estimated and may not be exact.

when the presence of selectable marker in the recombinant vaccinia virus is undesirable, such as for vaccine purposes ***(83)***. Other mechanisms of selection include: antibiotic resistance through the use of neomycin ***(84)*** or hygromycin ***(85)***, changes in plaque size ***(86,87)*** or red blood cell agglutination phenotypes ***(88)***, and alterations of host range ***(89–91)***.

Because homologous recombination occurs with a low frequency, and most selection methods allow for growth of some parental virus, plaques must be screened to identify the ones containing recombinant virus. By far the easiest method involves use of the *E. coli lacZ* gene, which has been included in many transfer vectors. This provides a positive colorimetric assay for the identification of recombinant viruses through the production of β-galactosidase (β-gal) ***(76)*** (**Fig. 2A**). A more recent colorimetric assay has been developed based on the *E. coli gusA* gene encoding [β-glucuronidase (GUS)], which is significantly smaller in size making plasmid and cloning manipulations somewhat easier ***(92)***. The *E. coli lacZ* or *gusA* genes can be used alone or in conjunction with one of the selection markers described above. However, in the absence of a colorimetric screening approach, plaques containing recombinant virus can be identified either by DNA or immunological analyses. The presence of DNA containing the foreign gene can be identified by DNA dot blot or polymerase chain reaction (PCR) analyses ***(60)***. Alternatively, the gene product can be identified by Western blot, immunoprecipitation, or immunostaining if an antibody is available.

The purpose of this protocol is to provide a simple and detailed outline of the processes involved in the generation of a typical recombinant vaccinia virus. All manipulations with live vaccinia virus and virus infected cells should be performed in a biological safety cabinet using sterile techniques. Waste should be decontaminated chemically or by autoclaving before disposal. In addition, vaccination of laboratory workers may be required by some institutions. However, the highly attenuated vaccinia virus strains MVA and NYVAC have been approved by the U. S., National Institutes of Health intramural biosafety committee for use without a biological safety cabinet or vaccination. Up-to-date detailed methodology, in a similar format as detailed in this protocol, for generation and characterization of recombinant MVA has recently been reported ***(93)***. For simplicity, the outlined protocols describe the procedures performed when utilizing vaccinia virus transfer vectors which provide for selection via the tk^- phenotype or acquisition of the *Ecogpt* gene (XGPRT selection) in the context of the wild-type vaccinia virus strain WR. Also included are the steps performed to identify recombinant vaccinia viruses that express the *E. coli lacZ* gene. Several other screening protocols are also included and the reader is encouraged to review the many alternative methods now available and cited here. These protocols can be used for identification of recombinant vaccinia viruses, as well as for characterization of the foreign gene product.

2. Materials

2.1. Cell Culture

1. Cell lines: HeLa [American Type Culture Collection (ATCC) (ATCC #CCL 2); HeLa S3 (ATCC #CCL 2.2); BS-C-1 (ATCC #CCL 26); CV-1 (ATCC #CCL 70); HuTK$^-$143B (ATCC #CRL 8303)].
2. Cell culture media: Eagle's minimal essential medium (MEM); Dulbecco's modified Eagle's MEM (DMEM); MEM spinner medium (Quality Biologicals, Gaithersburg, MD).
3. Cell culture supplements: fetal bovine serum (FBS); horse serum (HS); 2 m*M* L-glutamine (100X); 50 mg/mL gentamicin sulfate in water (1000X; stable at room temperature); 5 mg/mL 5-bromodeoxyuridine (BrdU) in water (200X; filter sterilize, store in the dark at –20°C); Saline A (350 mg/L $NaHCO_3$, 400 mg/L KCl, 8 g/L NaCl) containing 0.1% dextrose, 0.002% phenol red, 0.25% trypsin, and 0.02% EDTA for passage of monolayer cell lines.
4. Complete media prepared from the above reagents: MEM containing 10% FBS, glutamine, and gentamicin (MEM-10); MEM containing 2.5% FBS, glutamine, and gentamicin (MEM-2.5); DMEM containing 10% FBS, glutamine, and gentamicin (DMEM-10); MEM spinner medium containing 5% HS, glutamine, and gentamicin (MEM-S-5).
5. Specialized equipment for HeLa spinner cultures: 100- or 200-mL vented spinner bottles and caps with filters (#1965 series, and Bellco Biotechnology, Vineland, NJ).

2.2. Vaccinia Virus Growth, Titering, and Purification

1. Vaccinia virus: Wild-type strain WR (ATCC #VR1354), stable at –70 °C.
2. Solutions and buffers: 2.5 mg/mL trypsin (2X crystallized and salt-free, Worthington Biochemical, Freehold, NJ), filter sterilize, stable >1 yr at –20°C); 10 m*M* and 1 m*M* Tris-HCl, pH 9.0 (filter sterilize, store at room temperature); 36% w/v sucrose solution in 10 m*M* Tris-

HCl, pH 9.0 (filter sterilize, store at 4°C); 40%, 36%, 32%, 28%, and 24% (w/v) sucrose solutions in 1 m*M* Tris-HCl, pH 9.0 (filter sterilize, store at 4°C); 95% ethanol; 0.1% crystal violet in 20% ethanol (stable at room temperature).

3. Specialized equipment: Dounce homogenizer, glass and tight-fitting (Kontes Glass, Vineland, NJ); probe and/or cup sonicators (Misonix, Farmingdale, NY); 3- to 10-Liter vented spinner bottles and caps with filters (#1965 series and #A523-A59, Bellco Biotechnology).

2.3. DNA Transfection

1. Plasmid transfer vector containing the gene of interest.
2. Solutions and buffers: 2.5 *M* $CaCl_2$; transfection buffer [0.14 *M* NaCl, 5 m*M* KCl, 1 m*M* $Na_2HPO_4 \cdot 2H_2O$, 20 m*M* HEPES, 0.1% dextrose, pH 7.05 (HBS, filter sterilize, stable at –20°C)].

2.4. Selection and Screening of Recombinant Vaccinia Viruses

2.4.1. Production and Amplification of Virus Plaques

1. 2% low-melting-point (LMP) agarose (G-BRL, Grand Island, NY) in water (sterilized by autoclaving, stable at room temperature) (*see* **Note 1**).
2. 2X MEM containing 10% FBS and glutamine (2X MEM-10).
3. 10 mg/mL neutral red in water (100X; to filter sterilize, store a 4°C).
4. Cotton-plugged Pasteur pipets, autoclaved.
5. For *Ecogpt* selection:
   a. 10 mg/mL mycophenolic acid (MPA) in 0.1 *N* NaOH (400X; filter sterilize, store at –20°C).
   b. 10 mg/mL xanthine in 0.1 *M* NaOH (40X; filter sterilize, store at –20°C).
   c. 10 mg/mL hypoxanthine in water (670X; filter sterilize, store at –20°C).
6. For *tk*– selection:
   a. 5 mg/mL BrdU in water (200X; filter sterilize, store at –20°C in the dark).
7. For β-gal screening:
   a. 4% 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (Xgal) in *N*,*N*-dimethyl formamide (120X; store at 4°C).

2.4.2. Screening Virus Plaques by DNA Hybridization

1. Solutions and buffers: 0.4 *M* Tris-HCl, pH 7.5; 5 *N* NaOH; 5 *M* NaCl; 20X SSC (3 *M* NaCl, 0.3 *M* $Na_3citrate \cdot 2H_2O$); 10% (w/v) sodium dodecyl sulfate (SDS) in water; 5 mg/mL sheared salmon sperm DNA in water (store –20°C).
2. Dot- or slot-blot apparatus.
3. GeneScreen Plus (Dupont-NEN, Boston, MA) membrane (*see* **Note 2**).
4. Whatman 3MM filter paper.
5. ^{32}P-labeled DNA (probe).

2.4.3. Screening Virus Plaques by Western Blotting, Immunoblotting, Radioimmunoprecipitation, or Immunostaining

1. Polyclonal or monoclonal antibody to the protein of interest.
2. Cell lysis buffer (20 m*M* Tris-HCl, pH 8.0, 100 m*M* NaCl, 0.5% [v/v] Triton X-100 or N-40).
3. For Western or immunoblot:
   a. Phosphate buffered saline (PBS); PBS containing 0.5% (v/v) Tween-20 (PBS/Tween); PBS containing 0.5% (v/v) Tween-20, 0.2% sodium azide (w/v), and 4% (w/v) BSA or 1% (w/v) hydrolyzed gelatin.
   b. ^{125}I-labeled protein A, protein G, or appropriate second antibody (*see* **Note 2**).
   c. Nitrocellulose membrane; Whatman 3MM filter paper.
   d. Dot- or slot-blot apparatus; supplies and apparatus for performing SDS polyacrylamide gel electrophoresis (SDS-PAGE).
4. For radio-immunoprecipitation:
   a. [^{35}S]methionine (>1000 Ci/mmol) and/or [^{35}S]cysteine (>600 Ci/mmol).
   b. Methionine- and/or cysteine-free MEM; dialyzed FBS.
   c. Immobilized protein A or protein G Sepharose CL-4B, or agarose, beads.
   d. PBS containing 0.5% (v/v) Triton X-100.
5. For immunostaining virus plaques:
   a. Dulbecco's phosphate-buffered saline (DPBS), containing 2% (v/v) FBS.
   b. Horseradish peroxidase conjugated appropriate second antibody (*see* **Note 2**).
   c. O-Dianisidine (Sigma-Aldrich Co., St. Louis, MO).

   d. Hydrogen peroxide 30%, absolute ethanol, sterile (autoclaved) wooden toothpicks.

3. Methods

3.1. Preparation of Vaccinia Virus Stock

1. Maintain the HeLa S3 suspension cell line in MEM-S-5 at 37°C without CO_2. Count and passage the culture, at 1 to 2 d intervals as follows: when the culture density reaches $4–5 \times 10^5$ cells/mL, dilute to $1.5–2.5 \times 10^5$ cells/mL (*see* **Note 3**). Expand the culture when necessary.
2. One day prior to vaccinia virus infection, plate the HeLa spinner cells in monolayers as follows: Count cells and centrifuge for 5 min at 1800*g* at room temperature, use 5×10^7 cells for each 150-cm^2 flask (*see* **Notes** 4 and 5).
3. Resuspend cells to a final density of 2×10^6 cells/mL in MEM-10 (equilibrated to 37°C), dispense 25 mL/150-cm^2 flask, and incubate overnight at 37°C in a 5% CO_2 incubator.
4. Just prior to use, mix an equal volume of vaccinia virus stock (usually 1–2 × 109 pfu/mL) and 0.25 mg/mL trypsin (prepared from the 2.5 mg/mL trypsin stock) in a sterile tube and vortex vigorously (*see* **Note 6**). Incubate in a 37°C water bath for 30 min, vortexing at 5 to 10 min intervals. Sonicate the mixture in a cup sonicator in ice-water for 30 s.
5. Dilute the trypsinized virus in MEM-2.5 to $2.5–7.5 \times 10^7$ pfu/mL.
6. Aspirate the medium from the flasks containing the HeLa S3 cells and overlay with 2 mL of the diluted, trypsinized virus suspension (the optimal MOI is 1–3 pfu/cell). Incubate the flasks at 37°C in a CO_2 incubator for 2 h, rocking the flasks by hand at 15–30 min intervals to prevent drying of the monolayer.
7. Overlay the cells with 25 mL of MEM-2.5 and incubate for 3 d at 37°C in a CO_2 incubator.
8. Shake, thump, or scrape the flasks to loosen the cells, and pipet into sterile plastic screw-cap centrifuge tubes. Centrifuge for 10 min at 1800*g* at 4°C. Resuspend the cell pellets in MEM-2.5 (2 mL/5×10^7 cells). Disperse the cells by vortexing.
9. Lyse the cell suspension with three freeze-thaw cycles using a dry ice/ethanol bath and 37°C water bath. Disperse the cells by vortexing during each thaw. Sonicate the lysate in an ice-water filled cup sonicator for 30 s.
10. Aliquot and store the virus stock at –70°C. Volumes of 0.5 mL are convenient for later experiments. This virus stock can now be titered (*see* **Subheading 3.3**).

3.2. Purification of Vaccinia Virus

1. Just prior to use, mix equal volumes of vaccinia virus stock and 0.25 mg/mL trypsin (prepared from the 2.5 mg/mL trypsin stock); vortex. Incubate 30 min at 37°C, vortexing at 10 min intervals.
2. Count the HeLa S3 spinner culture cells. Remove 5×10^8 cells for each liter to be infected (*see* **Note 4**). Centrifuge cells for 10 min at 1800*g* at room temperature. Resuspend cells in MEMS-5 to a final density of 2×10^7 cells/mL. Transfer to a sterile Erlenmeyer flask (50–200 mL) containing a plastic stir bar and a cotton stopper.
3. Add the trypsinized virus to a MOI of 5–8 pfu/cell. Stir gently for 30 min at 37°C. Transfer cells to a vented spinner flask containing MEM-S-5 equilibrated to 37°C (1 L/5×10^8 cells) and stir for 3 d at 37°C.
4. Harvest the cells by centrifugation for 10 min at 1800*g* at 4°C. Resuspend in 10 m*M* Tris-HCl, pH 9.0 (14 mL/5×10^8 cells). Keep cell suspension on ice for remainder of the protocol or the infected cell suspension may be frozen at this stage (–70°C) after a quick freeze in a dry ice ethanol bath for longer term storage until purification steps are resumed.
5. Homogenize the cell suspension with 30–40 strokes in a tight-fitting, glass Dounce homogenizer. Examine a sample of the lysed cells for breakage by light microscopy using the trypan blue dye exclusion technique. Transfer the cell suspension to a sterile plastic screw-cap centrifuge tube or bottle. If necessary, the cell suspension may be stored at –70°C after a quick freeze in a dry ice ethanol bath.
6. Centrifuge the lysed cells for 10 min at 300*g* at 4°C to remove nuclei. Save the supernatant (virus stock) on ice in a sterile 50 mL plastic screw-cap centrifuge tube. Resuspend the cell pellet in 10 m*M* Tris-HCl, pH 9.0 (3 mL/5×10^8 cells), and centrifuge for 10 min at 300*g* at 4°C.

Combine with the previous supernatant and keep on ice.

7. Sonicate the virus stock using a probe sonicator as follows:
   a. Sterilize the probe by dipping it in 95% ethanol and passing it through a flame.
   b. Let probe cool.
   c. Remove cap from tube containing the virus stock and place probe into the virus stock.
   d. Sonicate at full power for 15 s.
   e. Wait 15 s and repeat sonication four times.

   If a probe sonicator is unavailable, sonication can be performed using a cup (*see* **Note 7**).
8. Layer the sonicated virus stock onto a cushion of 17 mL of 36% sucrose (in 10 m*M* Tris-HCl, pH 9.0) in a sterile SW-27 centrifuge tube. Centrifuge for 80 min at 32,900*g* (13,500 rpm in SW-27 rotor) at 4°C. Aspirate to remove supernatant; virus is in the pellet.
9. Resuspend the viral pellet in 1 mL of 1 m*M* Tris-HCl, pH 9.0. Sonicate once for 15 s with a probe sonicator or 1 min in a cup sonicator (*see* **step 7**). At this point the virus is substantially concentrated and purified away from host cell components and for general laboratory use the purification protocol may be shortened by proceeding to **step 15**. If further purification is desired (for example: virus intended for use in animals), proceed to **step 10**.
10. Prepare sterile 24–40% continuous sucrose gradients in sterile SW-27 centrifuge tubes the day before needed by carefully layering 6.8 mL of each sucrose solution (in 1 m*M* Tris-HCl, pH 9.0) in the following order: 40%, 36%, 32%, 28%, and 24%. Place the gradients at 4°C overnight.
11. Carefully overlay each sucrose gradient with 1 mL of the sonicated viral suspension from **step 9**. Centrifuge for 50 min at 26,000*g* (12,000 rpm in SW-27 rotor) at 4°C.
12. After centrifugation the virus appears as a milky band in about the middle of the gradient. Carefully aspirate to remove the sucrose above the virus band; discard. Carefully collect the virus band (about 10 mL) with a sterile pipet and place in a sterile screw-cap plastic centrifuge tube on ice.
13. Aspirate the remaining sucrose from the tube and recover the pellet containing aggregated virus from the bottom of the tube. Resuspend in 1 mL of 1 m*M* Tris-HCl, pH 9.0 by pipetting; sonicate as in **step 7**.
14. Repeat the virus banding procedures in **steps 10** through **12** with the viral pellet from **step 13.** Combine this viral band (about 10 mL) with the previous one from **step 12** and add 2 vol of 1 m*M* Tris-HCl, pH 9.0; vortex. The total volume should be about 60 mL. Transfer to sterile SW-27 centrifuge tubes and centrifuge for 60 min at 32,900*g* (13,500 rpm in SW-27 rotor).
15. Aspirate the supernatants and resuspend the virus pellets in 1 m*M* Tris-HCl pH 9.0 (0.5–1.0 mL/5 × 10^8 infected cells) (*see* **Note 4**). Sonicate in cup sonicator, divide into 0.25 mL aliquots, and store at –70°C. The purified virus stock can now be titered (*see* **Subheading 3.3**).

3.3 Titration of Vaccinia Virus Stocks

1. Prepare six-well (35 mm diameter) tissue culture plates of BS-C-1 cells by seeding 5 × 10^5 cells/well in a total volume of 2 mL of MEM-10. Do not swirl the plates as this results in clumping of the cells in the middle of the well. Incubate overnight at 37°C in a 5% CO_2 atmosphere to reach confluence (*see* **Note 8**).
2. For titration of a vaccinia virus stock, trypsinize as described in **step 4** of Methods **Subheading 3.1.** For titration of a purified virus stock, trypsinization is not required; however, the purified stock should be sonicated using a cup sonicator.
3. Prepare eight 10-fold serial dilutions, beginning with a 10^{-2} dilution, of the virus stock in MEM-2.5, using a fresh pipet for each dilution. This is most easily done by aliquoting 2.7 mL of MEM-2.5 into tubes 2 through 9 and 3 mL into tube 1. Remove 30 µL of medium from tube 1 and add 30 µL of the virus stock. Vortex to mix. The serial dilutions are then prepared by the sequential passing of 0.3 mL (note: for titration of purified virus stocks prepare nine 10-fold serial dilutions).
4. Aspirate the medium from the six-well cultures of BS-C-1 cells and infect the cell monolayers in duplicate with 1 mL aliquots of the 10^{-7}, 10^{-8}, and 10^{-9} dilutions (note: for titration of purified virus stocks plate the 10^{-8}, 10^{-9}, and 10^{-10} dilutions). Incubate 1–2 h at 37°C in a 5% CO_2

atmosphere, rocking the plates at 15 min intervals to prevent drying of the monolayer.

5. Overlay each well with 2 mL MEM-2.5 and incubate 2 d at 37°C in a 5% CO_2 atmosphere.
6. Aspirate the medium and add 0.5 mL of 0.1% crystal violet solution to each well. Incubate 5 min at room temperature, and then aspirate. Keeping the lids of the plates off, rest the plates on their lids at an angle in the biological safety cabinet to air dry.
7. Determine the virus titer by counting plaques in both wells, dividing by 2, and multiplying by the dilution factor of those wells. Most accurate results are obtained from wells with 20–80 plaques. Remember to take into account the 1:1 dilution of the virus stock and trypsin.

3.4. Infection and Transfection

1. Prepare a 25-cm^2 flask of CV-1 cells by seeding 10^6 cells in 4 mL of MEM-10 and incubating overnight at 37°C in a 5% CO_2 atmosphere. This will usually result in a culture that is just reaching confluence the next day.
2. Prepare an aliquot of trypsinized virus (usually WR strain) as in **step 4** of Methods **Subheading 3.1**.
3. Dilute the trypsinized virus in MEM-2.5 to 1.5×10^5 pfu/mL. Aspirate the medium from the flask of CV-1 cells and infect with 1 mL of the diluted virus (this yields a MOI of 0.05 pfu/cell). Incubate the cells for 2 h at 37°C in a 5% CO_2 atmosphere, rocking the flask by hand at 15 min intervals to prevent drying of the monolayer.
4. At 30 min prior to the end of the infection period, prepare the transfection mixture as follows: place 1 mL of transfection buffer (HBS) into a 12 × 75-mm polystyrene tube and add 5–10 µg of the recombinant transfer vector DNA (the volume of DNA should be no more than 50 µL), mix by gently tapping the tube two or three times, slowly add 50 µL of 2.5 *M* $CaCl_2$ drop-wise to the DNA solution, and again mix by gently tapping the tube two or three times. Incubate the mixture for 20–30 min at room temperature; a fine milky precipitate should appear (*see* **Note 9**).
5. Aspirate the virus inoculum and overlay the cell monolayer with the transfection mixture from **step 4**. Incubate for 30 min at room temperature. Add 9 mL of MEM-10 and incubate 3.5 h at 37°C in a 5% CO_2 atmosphere.
6. Aspirate the medium, add 10 mL of fresh MEM-10, and incubate for 2–3 d at 37°C in a 5% CO_2 atmosphere until the entire monolayer of cells is infected from the spreading virus.
7. Harvest the cells by scraping with a sterile disposable cell-scraper or rubber policeman and transfer to a sterile 15 mL plastic screw-cap centrifuge tube. Centrifuge for 10 min at 1800*g* at 4°C. Aspirate the supernatant, and resuspend the cell pellet in 1 mL of MEM-2.5.
8. Lyse the cell suspension with three freeze-thaw cycles as described in **step 9** in Methods **Subheading 3.1.** Store the lysate at –70°C if not used immediately.

3.5. Selection of Recombinant Vaccinia Viruses

1. Prepare six-well (35 mm diameter) tissue culture plates of BS-C-1 cells (for XGPRT selection) or HuTK$^-$143B cells (for *tk*– selection) by seeding 5×10^5 cells/well in a total volume of 2 mL of MEM-10. Do not swirl the plates as this results in clumping of the cells in the middle of the well. Incubate the cultures overnight at 37°C in a 5% CO_2 atmosphere to reach confluence (*see* **Note 8**).
2. For XGPRT selection, preincubate the cell culture monolayers for 12 to 24 h in MEM-2.5 containing 25 µg/mL MPA, 250 µg/mL xanthine, and 15 µg/mL hypoxanthine at 37°C in a 5% CO_2 atmosphere.
3. Thaw and sonicate the transfected cell lysate (from **step 8** in Methods **Subheading 3.4.**) for 30 s in an ice-water filled cup sonicator.
4. Prepare four 10-fold serial dilutions (10^{-4} to 10^{-1}) of the sonicated cell lysate in MEM-2.5. For XGPRT selection, MPA, xanthine, and hypoxanthine are included in the serial dilutions at the concentrations indicated in **step 2** above.
5. Aspirate the medium from the six-well cell cultures (**step 1** above) and infect the cell monolayers in duplicate with 1 mL aliquots of the 10^{-2}, 10^{-3}, and 10^{-4} dilutions. Incubate 1–2 h at 37°C in a 5% CO_2 atmosphere, rocking the plates at 15 min intervals.

6. Before the end of the infection period, melt a bottle of sterile 2% LMP agarose and place in a 42–45°C water bath to equilibrate. Equilibrate a bottle of 2X MEM-10 in the 42–45°C water bath.
7. Prepare 25 mL of the agarose overlay for each six-well plate as follows: for XGPRT selection, mix 12.5 mL of 2X MEM-10 and 12.5 mL of melted 2% LMP agarose (both equilibrated to 42–45°C) in a tube and add MPA, xanthine, and hypoxanthine to the final concentrations noted in **step 2** above. Mix by gently swirling or inverting the tube. For *tk*⁻-selection mix 12.5 mL of 2X MEM-10 and 12.5 mL of melted 2% LMP agarose to a tube and add 125 µL of 5 mg/mL BrdU (*see* **Note 10**) and mix by gently swirling or inverting the tube.
8. Remove the virus inoculum, overlay each well with 4 mL of the appropriate agarose overlay mixture, swirl the plates to mix and allow to solidify at room temperature or briefly at 4°C. Incubate for 2 d at 37°C in a 5% CO_2 atmosphere.
9. After the 2 d incubation period, prepare a second agarose overlay by mixing equal volumes of melted 2% LMP agarose with 2X MEM-10 (both equilibrated to 42–45°C). Add neutral red to a final concentration of 100 µg/mL, mix by gently swirling or inverting the tube. If β-gal screening is used, include 1/120 vol of 4% Xgal. Overlay each well with 2 mL of the second agarose preparation, allow to solidify, and incubate the plates at 37°C in a 5% CO_2 atmosphere until plaques can be easily visualized (6 h to overnight). Plaques will appear as clear areas surrounded by a red background. Plaques containing β-gal producing virus will appear blue due to hydrolysis of the Xgal substrate.
10. When virus plaques are readily detectable, either by the neutral red stain (which visualizes all plaques) or by the Xgal stain (which identifies β-gal producing plaques), prepare a set of sterile microcentrifuge tubes containing 0.5 mL of MEM-2.5 (preferably screw-cap tubes). Using sterile, cotton-plugged Pasteur pipets, and a rubber bulb (*see* **Note 11**), pick well-separated plaques by squeezing the bulb, piercing through the agarose to the bottom of the well, scraping the monolayer, and aspirating the agarose plug containing infected cells into the pipet. Transfer the plug to a tube containing 0.5 mL of MEM-2.5. The number of plaque isolates picked depends on the selection protocol utilized. For recombinant viruses encoding the *lacZ* or *Ecogpt* gene (for example: pSC11 or pTKgptF1s, respectively; *see* **Table 1** and **Fig. 2**) at least 6–12 plaques should be picked and screened. For recombinant viruses having *tk*⁻ selection only (for example, pSC59, *see* **Table 1**), 15–30 plaques should be picked due to the high rate of spontaneous *tk*⁻ mutations (*see* **Note 12**).
11. After picking the plaques, vortex to mix and perform three freeze-thaw cycles as described in **step 9** of Methods **Subheading 3.1.** Store the virus isolates at –70°C. If *tk*⁻ selection is used, then the virus isolates should be screened by one of the methods described in Methods **Subheading 3.6.** or mentioned in **Note 2**. After identifying plaques containing the recombinant vaccinia virus, proceed to **step 12** below. If β-gal screening or XGPRT selection is used, no further analysis of the plaques is required at this time.
12. Plaque purify the recombinant vaccinia virus isolates as follows. Prepare monolayers of an appropriate cell line as described in **steps 1** and **2**; one six-well plate for each plaque isolate (note that as with β-gal or XGPRT selected isolates, only a few *tk*⁻ isolates need to be plaque purified at this point).
13. Thaw the virus isolates and sonicate in an ice-water filled cup sonicator as described in **step 9** of Methods **Subheading 3.1.** Prepare three 10-fold serial dilutions (beginning at 10^{-1}) of each of the isolates. If XGPRT selection is used, preincubate cell monolayers with selective drugs and add selective drugs to serial dilutions of virus.
14. Aspirate the medium from the six-well plates and infect the monolayers in duplicate with 1-mL aliquots of the 10^{-1}, 10^{-2}, and 10^{-3} dilutions from **step 13**. Incubate 2 h at 37°C in a 5% CO_2 atmosphere, rocking by hand at 30 min intervals.
15. Repeat **steps 6** through **10**, for three rounds of plaque purification to ensure a clonally pure

recombinant vaccinia virus. Store the final recombinant vaccinia virus at –70°C. Proceed to Methods **Subheading 3.7.**

3.6. Amplification and Screening of Recombinant Vaccinia Virus Plaque Isolates

3.6.1. Amplification of Plaque Isolates

1. Amplify each plaque isolate on cell monolayers as follows: Prepare BS-C-1 cells (for XGPRT selection) or HuTK$^-$143B cells (for *tk*$^-$ selection) in 12- or 24-well tissue culture plates by seeding 1.25×10^5 or 2.5×10^5 cells/well, respectively. Incubate at 37°C in a 5% CO_2 atmosphere until confluent (usually overnight). If XGPRT selection is used, preincubate the cell monolayers 12 to 24 h in MEM-2.5 containing MPA, xanthine, and hypoxanthine (**step 2**, Methods **Subheading 3.5.**). It is also recommended that a monolayer of cells be infected with the parental vaccinia virus, and a monolayer of cells be left uninfected. These samples will be useful negative controls during later screening processes.
2. Infect individual wells containing confluent cell monolayers with 0.25 mL of each sonicated plaque isolate. For XGPRT selection carry out infection in the presence of MPA, xanthine, and hypoxanthine; for *tk*$^-$ selection carry out infection in the presence of BrdU. Incubate the plates for 2 h at 37°C in a 5% CO_2 atmosphere, rocking by hand at 15 min intervals.
3. Overlay each well with 0.5 mL of MEM-2.5 containing the appropriate drugs and incubate the plates 2 to 3 d at 37°C in a 5% CO_2 atmosphere or until cytopathic effect (cell rounding) is evident throughout the monolayer (*see* **Note 13**). At this point the treatment of the amplified plaque isolates will vary depending on what screening method will be employed. Five examples of methods for analysis of plaques are given in the next sections and further examples are mentioned in **Note 2.**

3.6.2. Detection of Recombinant Vaccinia Virus by DNA Hybridization

1. After complete cytopathic effect is observed during the amplification of plaque isolates (*see* **Note 13**), as described in **step 3** of Methods **Subheading 3.6.1.**, harvest the cells in each well by scraping and transfer to microcentrifuge tubes. Centrifuge the cells at full speed in a microcentrifuge for 5 min, and aspirate the medium. Resuspend the cell pellets in 0.5 mL PBS, perform three freeze-thaw cycles as described in **step 9** of Methods **Subheading 3.1.**, and place on ice.
2. Cut a section of the GeneScreen Plus membrane and two sections of Whatman 3MM filter paper to fit the dot- or slot-blotting apparatus, and soak in a tray containing 0.4 *M* Tris-HCl, pH 7.5 for 30 min.
3. Transfer 100 µL of each lysate to a new microcentrifuge tube, and denature the DNA by addition of 5 µL of 5 *N* NaOH (final concentration of 0.25 *N* NaOH). Vortex to mix and incubate 10 min at room temperature.
4. Chill the denatured DNA on ice.
5. Dilute the denatured DNA with 200 µL of 0.125 *N* NaOH, 0.125X SSC.
6. Sonicate the diluted denatured DNA in an ice-water-filled cup sonicator, and store on ice.
7. Assemble the dot- or slot-blotting apparatus with the presoaked membrane and filter paper.
8. Add 100 µL of each DNA sample in duplicate to the wells of the apparatus.
9. Allow solutions to remain on the membrane without any suction for 30 min.
10. After 30 min, apply a slight suction to the apparatus until all liquid has passed through the membrane.
11. Remove the membrane and air dry at room temperature.
12. Denature an aliquot of the 5 mg/mL sheared salmon sperm DNA stock by heating 3 min at 100°C and chilling on ice. Prehybridize the membrane for 30 min at 65°C by incubation in 10 mL of 1% SDS, 1 *M* NaCl containing 200 µg/mL denatured salmon sperm DNA in a sealable plastic bag.
13. Add 0.5–1.0 mL of 1% SDS, 1 *M* NaCl containing 200 µg/mL denatured salmon sperm DNA, and 100 ng of ^{32}P-labeled probe DNA (approx $1–4 \times 10^7$ dpm/µg) (*see* **Note 14**). Reseal the bag and incubate with constant agitation for 6–24 h at 65°C.
14. Remove the membrane from the bag and wash as follows:

   a. 2 times with 100 mL of 2X SSC at room temperature for 5 min.
   b. 2 times with 200 mL of 2X SSC containing 1% SDS at 65°C for 30 min.
   c. 2 times with 100 mL of 0.1X SSC at room temperature for 30 min. All washes should be performed with constant agitation.
15. Place the membrane with the DNA face up on a sheet of filter paper to adsorb excess liquid, wrap in plastic wrap, and expose to X-ray film. Plaque isolates containing recombinant virus are identified by hybridization with the probe DNA.
16. When one or several plaque isolates are identified, proceed with the plaque purification steps starting at **step 12** of Methods **Subheading 3.5.**

3.6.3. Detection of Recombinant Vaccinia Virus by Immunoblotting of the Recombinant Gene Product

1. After complete cytopathic effect is observed during the amplification of plaque isolates (*see* **Notes 13** and **15**), as described in **step 3** of Methods **Subheading 3.6.1.,** harvest the cells in each well by scraping and transfer to a set of microcentrifuge tubes. Centrifuge the cells at full speed in a microcentrifuge for 5 min, and aspirate the medium (recover the supernatants if the protein of interest is secreted). Resuspend the cell pellets in 0.5 mL PBS and perform three freeze-thaw cycles, as described in **step 9** of Methods **Subheading 3.1.,** sonicate in a cup sonicator, and place on ice.
2. Cut a section of nitrocellulose and two sections of Whatman 3MM filter paper and soak them in distilled water.
3. Assemble the dot- or slot-blot apparatus and apply 50 µL of each lysate into individual wells (in duplicate). If the protein of interest is secreted, the medium from the infected cell monolayers can be substituted for the cell lysates.
4. Allow lysates to remain on the membrane without any suction for 30 min.
5. After 30 min, apply a slight suction to the apparatus until all liquid has passed through the membrane.
6. Soak the membrane in 50 mL of PBS/Tween containing 4% BSA or 1% hydrolyzed gelatin for 30 min to 1 h. Plastic lids from micropipet tip racks work well as washing trays.
7. Wash the membrane in 50–100 mL of PBS/Tween; dilute the antibody to the foreign protein in PBS/Tween (as appropriate for the antibody) using a minimal volume (just enough to cover the membrane). Pour off the wash solution, replace with the antibody solution, and incubate for at least 1 h at room temperature or overnight at 4°C with gentle rocking.
8. Wash the membrane with four changes of PBS/Tween (50–100 mL/wash; 15–20 min/wash); dilute ^{125}I-labeled protein A, protein G, or appropriate second antibody in a minimal volume of PBS/Tween. Pour off the wash solution, replace with the radiolabeled solution, and incubate for at least 1 h at room temperature.
9. Pour off the radiolabeled solution and wash the membrane with four changes of PBS/Tween as in **step 8**. Blot the membrane on filter paper to remove excess liquid, wrap in plastic wrap and expose to X-ray film. Develop the autoradiograph and determine which amplified plaque isolates contain recombinant virus producing the protein of interest.
10. When one or several recombinant virus plaque isolates are identified, proceed with the plaque purification steps starting at **step 12** of Methods **Subheading 3.5.**

3.6.4. Detection of Recombinant Vaccinia Virus by Western Blotting of the Recombinant Gene Product

1. After complete cytopathic effect is observed during the amplification of plaque isolates (*see* **Notes 13** and **15**), as described in **step 3** of Methods **Subheading 3.6.1.**, harvest the cells in each well by scraping and transfer to a set of microcentrifuge tubes. Centrifuge the cells at full speed in a microcentrifuge for 5 min, and aspirate the medium. Alternatively, if the protein of interest is secreted, the supernatants can be recovered and either analyzed directly or first concentrated by immuno-precipitation or use of a microconcentrator. Lyse the cell pellets by resuspending in 200 µL of cell lysis buffer (*see* Materials **Subheading 2.4.3.** and **Note 16**), vortex, and incubate on ice for 15 min. Centrifuge the cell lysates for 5 min at

full speed to remove nuclei and debris, and transfer the supernatants to clean tubes.

2. Prepare an SDS-PAGE gel(s).
3. Aliquot 20 μL of each lysate into a new microcentrifuge tube, add 20 μL of 2X SDS protein gel sample buffer, and heat 100°C for 3 min. Centrifuge 1 min at top speed in a microcentrifuge. Load 25–30 μL of each sample into the wells of the gels and separate the proteins by electrophoresis.
4. Transfer the separated proteins electrophorectically onto a sheet of nitrocellulose membrane using a transfer apparatus.
5. Carry out **steps 6** through **10** of Methods **Subheading 3.6.3.**

3.6.5. Detection of Recombinant Vaccinia Virus by Radioimmuno-precipitation of the Recombinant Gene Product

1. Amplify the plaque isolates as described in Methods **Subheading 3.6.1., steps 1** through **3** (*see* **Notes 13** and **15**). However, after 1–2 d postinfection proceed as follows.
2. Aspirate the medium from each well and wash two times with 2 mL of methionine- and/or cysteine-free MEM containing 2.5% dialyzed FBS.
3. Remove the final wash and overlay each well with 0.5 mL of methionine- and/or cysteine-free MEM containing 2.5% dialyzed FBS and 50–100 μCi/mL of [^{35}S]methionine (>1000 Ci/mmol) and/or [^{35}S]cysteine (>600 Ci/mmol). Incubate for an additional 24 h at 37°C in a 5% CO_2 atmosphere.
4. Add 100 μL of MEM-2.5 to each well for 1 h at 37°C in a 5% CO_2 atmosphere. Aspirate the radioactive supernatants (recover and save the supernatants if the protein of interest is secreted), overlay each well with 0.5 mL PBS, scrape, and transfer to a set of microcentrifuge tubes. Centrifuge the cells for 5 min at full speed, aspirate the PBS, and lyse the cell pellets in 200 μL of cell lysis buffer (*see* Materials **Subheading 2.4.3.** and **Note 16**). Vortex and incubate on ice for 15 min. Centrifuge the cell lysates for 5 min at full speed to remove nuclei and debris and transfer the supernatants to new tubes.
5. Aliquot 10–50 μL of each metabolically labeled cell lysate (use 100–500 μL of each supernatant if the protein of interest is secreted) to a set of microcentrifuge tubes.
6. Add 100 μL of PBS-Triton X-100 containing the appropriate dilution of antibody to the foreign protein (as appropriate for the antibody). Incubate the tubes for 2 h at room temperature or 4°C overnight.
7. Add a 50 μL aliquot of a 20% (v/v) suspension of immobilized protein A or protein G Sepharose CL-4B, or agarose, beads [as appropriate for the antibody ***(94)***]. If necessary, a second antibody with specificity to the species of the first antibody can be included as well. Rotate the tubes for 1 h at 4°C.
8. Centrifuge the tubes for 5 min at 500*g* (a swinging bucket rotor works best to create a small pellet; alternatively use a microcentrifuge). Aspirate the supernatants with a round gel-loading micropipet tip taking care not to touch the beads.
9. Wash the beads two times as follows: add 1.0 mL of PBS-Triton X-100 to each tube, shake to mix, and repeat **step 8** (*see* **Note 17**).
10. Resuspend the pellet in 20 μL of 2X SDS sample buffer and heat 100°C for 3 min. Centrifuge for 5 min at top speed in a microcentrifuge.
11. Prepare SDS-PAGE gel(s).
12. Load 20 μL of each sample into the wells of the gels and separate the proteins by electrophoresis.
13. Process the SDS-PAGE gels for detection of labeled proteins by fixation, amplification, and fluorography.
14. When one or several recombinant virus plaque isolates are identified proceed with the plaque purification steps starting at **step 12** of Methods **Subheading section 3.5.**

3.6.6. Detection of Recombinant Vaccinia Virus by Immunostaining of the Recombinant Gene Product

1. Prepare six-well (35 mm diameter) tissue culture plates of BS-C-1 cells (for XGPRT selection) or HuTK$^-$ 143B cells (for *tk*$^-$ selection) by seeding 5 × 10^5 cells/well in a total volume of 2 mL of MEM-10. Do not swirl the plates as this results in clumping of the cells in the middle of the well. Incubate the cultures overnight at 37°C in a 5% CO_2 atmosphere to reach confluence (*see* **Note 8**).

2. For XGPRT selection, preincubate the cell culture monolayers for 12 to 24 h in MEM-2.5 containing 25 μg/mL MPA, 250 μg/mL xanthine, and 15 μg/mL hypoxanthine at 37°C in a 5% CO_2 atmosphere.
3. Thaw and sonicate the transfected cell lysate (from **step 8** in Methods **Subheading 3.4.**) for 30 s in an ice-water-filled cup sonicator.
4. Prepare four 10-fold serial dilutions (10^{-4} to 10^{-1}) of the sonicated cell lysate in MEM-2.5. For XGPRT selection, MPA, xanthine, and hypoxanthine are included in the serial dilutions at the concentrations indicated in **step 2** above.
5. Aspirate the medium from the six-well cell cultures (**step 1** above) and infect the cell monolayers in duplicate with 1 mL aliquots of the 10^{-2}, 10^{-3}, and 10^{-4} dilutions. Incubate 2 h at 37°C in a 5% CO_2 atmosphere, rocking the plates at 15 min intervals.
6. Aspirate the inoculum from the six-well cell cultures and overlay each well with 2 mL MEM-2.5 containing the appropriate selection drugs and incubate 2 d at 37°C in a 5% CO_2 atmosphere. Virus plaques should be visible by 48 h and the plates are ready to be immunostained.
7. Dilute the appropriate polyclonal antiserum (1:800 to 1:1000 usually a good starting point) in DPBS containing 2% FBS, or the appropriate monoclonal antibody. Calculate the total amount to be used needing 1 mL/well (*see* **Note 18**).
8. Aspirate medium from all wells; wash each well twice with 2 mL of D-PBS, carefully aspirating between washes, and add 1 mL of the antibody solution prepared in **step 7** to each well. Rock the plates slowly on rocking platform for 1 h at room temperature.
9. Dilute appropriate horseradish peroxidase conjugated secondary antibody 1:800 in DPBS-2%FBS. Calculate total amount needed using 1 mL per well.
10. Aspirate primary antibody solution from all wells; wash each well twice with 2 mL of DPBS, carefully aspirating between washes, no incubation between washes.
11. Add 1 mL of horseradish peroxidase conjugated secondary antibody solution to each well, rock plates gently on rocking platform for 1 h at room temperature. At 20 min prior to conclusion of secondary antibody incubation, use a small measuring spatula to place an approx 3 mm bead of O-dianisidine in the bottom of a 1.5 mL microcentrifuge tube and add 500 μL absolute ethanol and vortex briefly; then place tube in a 37°C water bath for 5–10 minutes, then vortex again, and centrifuge full speed for 30 s. This amount of O-dianisidine is not expected to devolve completely, a saturated solution is required.
12. Add 200 μL of this substrate stock solution to 10 mL D-PBS containing 10 μL of hydrogen peroxide 30% and mix well by vortexing.
13. Aspirate secondary antibody solution from each well and wash each well twice with 2 mL of DPBS-2%FBS. Add 600 μL of the substrate solution made in **step 12** per well. Incubate plate at room temperature (no rocking) for at least 10 min, then examine plate with the naked eye or using a microscope for the presence of orange-rust color foci. Plates should not be left in the substrate solution for longer than 30 min, at which time the cell monolayer begins to fall apart. Circle positive foci (orange-rust stained) on the backside of the plate.
14. Aspirate substrate solution from the wells, operate only on one plate at a time to avoid drying of the monolayers, multiple plates may be incubated in DPBS, not substrate solution. Use sterile toothpicks to scrape up the foci of cells and break off toothpick into a sterile 1.5 mL screw top microcentrifuge tube containing 500 μL of MEM-2.5. Pick all positive foci during this first-round of plaque purification.
15. After picking the plaques, vortex to mix and perform three freeze-thaw cycles as described in **step 9** of Methods **Subheading 3.1.** Store the virus isolates at –70°C. When one or several plaque isolates are identified, proceed with the plaque purification steps starting at **step 12** of Methods **Subheading 3.5.**

3.7. Final Amplification of a Recombinant Vaccinia Virus Plaque Isolate

1. Prepare a 25-cm^2 flask with the appropriate cell line: BS-C-1 cells (for XGPRT selection) or $HuTK^-143B$ cells (for *tk*⁻ selection) by seeding 1×10^6 cells and incubating at 37°C in a 5% CO_2 atmosphere until confluent (usually over-

night). If XGPRT selection is used, pre-incubate the cell monolayer for 12–24 h in MEM-2.5 containing MPA, xanthine, and hypoxanthine (**step 2,** Methods **Subheading 3.5.**).

2. Choose one or several of the plaque purified recombinant vaccinia virus isolates, thaw and sonicate in an ice-water-filled cup sonicator.
3. Infect the cell monolayer as follows: add 0.25 mL of one sonicated plaque isolate to a plastic centrifuge tube and add an additional 0.75 mL of MEM-2.5 and the appropriate selective drugs; remove the medium from the monolayer by aspiration; and overlay the monolayer with the diluted virus preparation. Incubate the flask for 2 h at 37°C in a 5% CO_2 atmosphere, rocking by hand at 15 min intervals.
4. Overlay the monolayer with 5 mL of MEM-2.5 containing the appropriate selective drugs and incubate the culture at 37°C in a 5% CO_2 atmosphere for 2–3 d or until cytopathic effect (cell rounding) is evident throughout the monolayer.
5. Harvest the cells by scraping, transfer to a sterile 15 mL screw-cap conical centrifuge tube, and centrifuge for 10 min at 1800*g* at 4°C. Aspirate the supernatant, resuspend the cell pellet in 0.5 mL of MEM-2.5, and perform three freeze-thaw cycles as described in **step 9** of Methods **Subheading 3.1.** The amplified recombinant virus stock can be stored at –70°C if not used immediately.
6. Scale up the virus stock by infecting a 150-cm^2 tissue culture flask containing a confluent monolayer of the appropriate cell line. To prepare the cells, scale up the procedure described in **step 1** above.
7. Infect the cell monolayer as follows: add 0.25 mL of sonicated virus stock from **step 5** to a plastic centrifuge tube and add an additional 2.75 mL of MEM-2.5 and the appropriate selective drugs; remove the medium from the monolayer by aspiration; overlay the monolayer with the diluted virus preparation. Incubate the flask for 2 h at 37°C in a 5% CO_2 atmosphere, rocking by hand at 15 min intervals.
8. Overlay the monolayer with 10 mL of MEM-2.5 containing the appropriate selective drugs and incubate the culture at 37°C in a 5% CO_2 atmosphere for 2–3 d or until cytopathic effect (cell rounding) is evident throughout the monolayer.
9. Harvest the cells by scraping; transfer to a sterile 15 mL screw-cap conical centrifuge tube; and centrifuge for 10 min at 1800*g* at 4°C. Aspirate the supernatant, resuspend the cell pellet in 2 mL of MEM-2.5, and perform three freeze-thaw cycles as described in **step 9** of Methods **Subheading 3.1.** The amplified recombinant virus stock can be stored at –70°C if not used immediately.
10. Count the HeLa S3 spinner cell culture; for each 150-cm^2 flask to be infected remove 5×10^7 cells and centrifuge for 5 min at 1800*g* at room temperature. (Usually five flasks of cells are prepared at this stage.)
11. Resuspend cells to a density of 2×10^6 cells/mL in MEM-10 equilibrated to 37°C and dispense 25 mL to each 150-cm^2 tissue culture flask. Incubate overnight at 37°C in a 5% CO_2 incubator.
12. Sonicate the virus stock from **step 9** above. For each 150-cm^2 tissue culture flask to be infected, dispense 0.25 mL of the virus stock (**step 9** above) and 2.75 mL MEM-2.5 into a 15 mL screw-cap conical centrifuge tube. (Selective drugs are not required at this stage.)
13. Aspirate the medium from the flasks containing the HeLa S3 cells and overlay with 3 mL of the diluted virus suspension. Incubate at 37°C in a CO_2 incubator for 2 h, rocking the flasks by hand at 15–30 min intervals to prevent drying of the monolayer.
14. Overlay the cells with 25 mL of MEM-2.5/flask and incubate for 3 d at 37°C in a CO_2 incubator.
15. Harvest the cells by shaking, thumping, or scraping the flasks, and pipet into sterile plastic screw-cap centrifuge tubes. Centrifuge for 10 min at 1800*g* at 4°C. Aspirate the supernatant and resuspend the cell pellet in 2 mL of MEM-2.5/150-cm^2 flask. Disperse the cells by vortexing and lyse with at least three cycles of freeze-thawing in a dry ice/ethanol bath and 37°C water bath. Vortex cells during each thaw. Sonicate the thawed lysate in an ice-water-filled cup sonicator for 30 s.
16. Store the recombinant virus preparation at –70°C. This virus stock can now be titered as detailed in Methods **Subheading 3.3.**

4. Notes

1. Contaminants in LMP agarose from some sources may be toxic to cells; we have found that the LMP agarose from G-BRL has been consistently suitable.
2. Variations in the DNA and immuno-based assays described in this article can be performed. For example, utilization of other membranes for immobilization of DNA and protein samples and utilization of nonradioactive-based detection mechanisms can be employed. Other methods for screening and analyzing recombinant vaccinia viruses include DNA analysis by Southern blot or PCR techniques and mRNA analysis by Northern blot techniques. For detailed protocols of these techniques see *ref.* ***13.*** A method of *in situ* immunostaining of virus plaques can be performed for cell surface-expressed or secreted recombinant proteins if an antibody or antiserum is available (*see* **Subheading 3.6.6.**). Also, a recent streamlined procedure in which single *tk*$^-$ recombinant plaque isolates can be obtained in 96-well cell culture plates directly, without the agarose overlays has been described ***(95).***
3. Maintain the density of the HeLa S3 spinner culture between 1.5–5 × 10^5 cells/mL. Culture viability drops off dramatically at higher densities and the cells do not grow well at densities below 1 × 10^5 cells/mL.
4. As a general guideline, the yield of vaccinia virus from a cell lysate of either HeLa S3 suspension or HeLa monolayer cultures is approx 5 × 10^8–4 × 10^9 pfu/mL when each 150–cm^2 flask of infected cells is resuspended into 2 mL of MEM-2.5. After purification of vaccinia virus by banding in sucrose each liter of 5 × 10^8 infected HeLa S3 cells yields 0.5–1 mL with a titer of approx 1–5 × 10^{10} pfu/mL.
5. Stocks of vaccinia virus can be prepared using the HeLa monolayer cell line in place of the HeLa S3 suspension cell line. This is convenient when smaller stocks of virus are required (20–40 mL of stock with titers of about 10^9 pfu/mL) or if equipment for growing spinner cells is not available.
6. Always perform trypsinization of vaccinia virus stocks just prior to use. Never store trypsinized viruses as this results in major losses in virus titer even at –70°C.
7. When purifying vaccinia virus and a probe sonicator is unavailable, split the cell lysate into 3 mL aliquots and sonicate each separately in an ice-water filled cup sonicator at full power for 1 min. Repeat sonication four times, with at least a 30 s interval of incubation on ice each time. Replenish the ice in the cup as required to maintain cold temperature.
8. It is important that the density of the cells in the monolayer not be too high when plaqueing virus as this may result in small plaque size and/or deterioration of the cell monolayer. It is best to use monolayers of cells in which have just reached confluence (10^6 cells/35 mm well tissue culture dish) for HuTK$^-$, CV-1, or BS-C-1 cells. This can usually be achieved by seeding 5 × 10^5 cells per well (35 mm diameter) in a ßtotal volume of 2 mL of medium the morning of the day prior to use.
9. The inclusion of wild-type vaccinia DNA in the transfection preparation yields a higher efficiency of recombination. This is accomplished by adding 1 µg of wild-type vaccinia DNA with the transfer vector DNA containing the cloned gene of interest. Also, alternative DNA transfection protocols such as those using Lipofectin (G-BRL), DOTAP (Boehringer Mannheim, Indianapolis, IN), or Transfectam (Promega, Madison, WI) can be utilized.
10. After thawing the BrdU stock, a 5–10 min incubation at 37°C followed by vortexing is required to ensure that the BrdU is in solution.
11. When picking plaques, move the pipett tip in a circular motion covering an area just slightly larger than the size of the plaque while maintaining contact with the bottom of the well. This will ensure good recovery of the infected cells in the plaque area. Also, the use of screw-cap microcentrifuge tubes ensures tight seals and prevents sample loss and contamination during the freeze-thaw cycles and manipulations.
12. When the *tk*$^-$ phenotype is used for selection without a concomitant screening protocol, it is important to pick 15–30 plaques because up to 80–90% of the plaques can be the result of spontaneous *tk*$^-$ vaccinia virus mutants. It is

critical to screen plaque isolates at this stage by one of the methods described in Methods **Subheading 3.6.**, or mentioned in **Note 2**, to identify positive recombinant viruses. After this initial recombinant virus identification is performed, only 6–8 plaques need be picked during the second and third rounds of plaque purification. It is usually necessary to purify only one or two of these isolates. Save the others until the final virus preparation has been made. If screening for the production of β-gal is used in conjunction with *tk*⁻ selection, or if XGPRT selection is employed, then only 6–12 plaques need be picked in the initial plaque purification step. A few of these can be immediately plaque purified. The presence of β-gal activity or MPA resistance is a very good indication that the virus isolate contains the inserted gene of interest.

13. When amplifying a series of plaque isolates (especially in the first round of *tk*⁻ selection), allow sufficient time (up to 3–4 d) for all or most of the individual monolayers to achieve a high degree of infection. Make note of which wells, if any, have little cytopathic effect; this will aid in assessing the positive signals obtained with DNA or immuno-based analyses. If minimal cytopathic effect is observed after 2 d, the cultures can be supplemented with fresh medium containing appropriate selective drugs and incubated further.
14. When screening amplified plaque isolates by DNA hybridization, prepare the ^{32}P-labeled probe from DNA containing the gene of interest and not the flanking vaccinia virus sequences as the latter will hybridize with all vaccinia virus samples. The DNA probe can be prepared via any commercially available nick-translation or random-priming kit.
15. The Western blot, immunoblot, and radioimmunoprecipitation assays outlined in Methods **Subheading 3.6.3.** through **3.6.5.** are intended for screening many plaque isolates in order to identify recombinant vaccinia viruses producing the protein of interest. When utilizing the hybrid vaccinia virus/T7 system, the cell monolayers must be coinfected with a recombinant vaccinia virus expressing T7 RNA polymerase such as vTF7-3 ***(21)*** (MOI of 1). Foreign genes controlled by early vaccinia promoters may yield a weaker signal in the immuno-based detection assays. However, all these assays can be used to characterize the protein produced by a recombinant vaccinia virus after it has been plaque purified and grown up as a working stock of virus.
16. When preparing cell lysates for analysis of the protein of interest, it may be necessary to add one or several protease inhibitors to the cell lysis buffer. Phenylmethylsulfonyl fluoride is commonly used at a final concentration of 0.2 m*M* (20 m*M* stock). Also, virus isolates producing a secreted recombinant protein can be screened by harvesting the medium from cells infected at any stage in the plaque amplification process. However, if the medium will be concentrated with microconcentrators, it is important to use serum free medium or only 1% serum as a high concentration of serum proteins interfere with SDS-PAGE analysis.
17. During the immunoprecipitation assays an additional wash with PBS containing 0.1% deoxycholic acid and 0.1% SDS can be performed to reduce nonspecific background. If background remains problematic, then the cell lysates can be precleared by performing a mock immunoprecipitation using preimmune sera or irrelevant antibody with the protein A or protein G beads. After pelleting these beads by centrifugation the precleared lysate is recovered and then used in the immunoprecipitation assay.
18. Virtually any specific antibody (polyclonal sera or monoclonal) can be used in the immunostaining protocol of virus plaques. The best amount of antibody giving good signal over background staining may have to be empirically determined. It is best to perform parallel immunostaining of a mock transfection preparation or other nonrecombinant virus as a negative control for comparison. The immunostaining protocol of live virus plaques can also be successfully used to screen and identify recombinant vaccinia viruses encoding soluble/secreted [i.e., non-membrane anchored soluble gp140 HIV-1 envelope glycoproteins

(96)] gene products, although the signal is often considerably less intense than that obtained when staining for surface-expressed gene products.

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